XLIX. THE UREASE CONTENT OF CERTAIN INDIAN SEEDS.

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During preliminary work on the nitrogenous constituents of certain Indian seeds a number were tested for urease.

Owing to the fact that they contain urease, soy beans have been utilised by Marshall [1913, 1, 2), and by Plimmer and Skelton [1914] for the estimation of urea in urine. In the present work it was thought worth while to test certain well defined varieties of soy-beans in order to see if there were any large variation in their urease activity. During the course of the work a seed was found whose urease activity was many times greater than that of soy-bean and this short paper has chiefly been written to bring this fact to notice, since it should be very useful in the estimation of urea.

Zemplèn [1912] has tested the seeds of many plants for urease. He found the enzyme in most papilionaceous but not in graminaceous ones.

In the work here described the following preliminary test for urease was applied to the seeds. They were finely powdered in a Maercker mill and about 0·5 g. was then placed in a test-tube containing 10 cc. of 1 per cent. urea solution with 3 drops of phenolphthalein and a few drops of toluene. Each tube was left 24 hours. If urease be present in the seed, ammonium carbonate should be formed and shew its presence by the reddening of the liquid.

By this method the following seeds appeared to contain urease :

Soy bean yellow, Soy bean spotted, Soy bean chocolate, Soy bean common, Soy bean black, *Sida spinosa*, Gram (*Cicer arietinum*), Rice (*Oryza sativa*), Cowa (*Setaria italica*), Sword bean (*Canavalia ensiformis*), (both a sample from U.S.A. and a sample grown at Dacca), *Urana lobata*, Khulti kalai (*Dolichos biflora*), Castor bean (*Ricinus communis*).

The method indicated the absence of urease in the following seeds: French beans (*Phaseolus vulgaris*), Velvet beans (*Mucuna pruriens*), Sona mung (*Phaseolus radiatus*), *Sida* sp. (from Burmah), *Sida cordifolia*, White til (*Sesamum indicum*), Black til (*Sesamum indicum*), Mati kalai (*Phaseolus mungo*), Cow pea (*Vigna catiang*), White mustard (*Brassica campestris* var. *sarson*), Red mustard (*Brassica campestris* var. *dichotoma*), Niger (*Guizotia abyssinica*), Taramira (*Eruca sativa*), Sweet pea (*Lathyrus sativus*).

The above test is by no means a reliable one, however, as it was found later (see below) that certain seeds which by the above test appeared to contain urease actually contained none at all, *e.g.*, cowa and rice. The shell of castor bean is strongly alkaline and hence itself colours the test solution red.

A number of the above seeds were next taken for determination of their comparative urease activity.

The following method was used:

10 g. of the powdered seed were treated with 100 cc. of distilled water and allowed to stand with occasional shaking for one hour at room temperature in presence of toluene. 2 cc. of this extract were then added to 50 cc. of a 1 per cent. urea solution together with 0·5 cc. of toluene. 5 cc. of the liquid were then immediately removed and titrated, using methylorange as indicator, with N/10 HCl. Thence onward, generally at half hour intervals, successive portions of 5 cc. were titrated. The tests were carried on at room temperature, about 27°.

We thus have a small amount of enzyme acting on a large amount of substrate and therefore the method is a fairly accurate comparative test of the urease activity. The tables shew that there are considerable differences in the urease activity of different seeds.

The following were in this way found to contain no trace of urease: *Sida spinosa*, Rice (*Oryza sativa*), Cowa (*Setaria italica*), Peas (*Pisum arvense*), Mati kalai (*Phaseolus mungo*), Gram (*Cicer arietinum*), Sona mung (*Phaseolus radiatus*), Cow pea (*Vigna catiang*), Velvet beans (*Mucuna pruriens*).

A number of pure line types of Mati kalai (*Phaseolus mungo*) grown by the Economic Botanist to the Government of Bengal, besides certain well-defined local varieties, were tested. Not one was found to contain a trace of urease.

It might be here mentioned that during the course of this work a ground sample of Khulti kalai (*Dolichos biflora*) which is seen in the table to contain urease was tested two weeks later and gave no indication whatever of its

cc. N/10 HCl *required.*

Time in hours	Soya beans						Sword beans		Khulti kalai (*Dolichos biflora*)	Urena Lobata	Urena Lobata, another sample
	Yellow	Common	Chocolate	Spotted	Black	Rymbsa Ktang	U.S.A.	Dacca			
initial	0·4	0·5	0·5	0·8	0·5	0·5	1·2	0·6	0·2	0·2	0·4
0·5	1·8	2·2	2·0	2·9	1·5	1·4	8·7	5·9	2·7	1·9	1·7
1	3·6	3·5	3·0	4·2	3·0	2·7	13·0	15·4	3·2	4·0	3·9
1·5	4·6	4·4	4·6	5·6	4·4	3·7	15·7	16·7	3·7	5·4	5·2
2	5·4	5·4	5·0	6·7	5·2	4·1	15·9	16·7	4·2	—	6·8
2·5	—	—	—	—	—	—	—	—	—	—	7·9
3·5	—	—	—	—	—	—	—	—	—	9·1	
4	—	8·5	7·7	10·3	7·9	7·7	16·0				
5	—	9·7	8·9	12·2	9·4	8·5	—	—	6·2		
6	—	11·5	10·0	13·6	9·4	9·2	16·0	—	7·2		
7·5	—	—	—	—	—	—	—	—	—	10·4	
14	15·9	16·5	16·7	16·5	16·4	16·1	—	—	—	15·8	
15	—	—	—	—	—	—	—	—	—	15·8	
24	—	—	—	—	—	—	—	—	—	—	13·3
26	—	—	—	—	—	—	—	—	—	—	14·3
50	—	—	—	—	—	—	—	—	—	—	15·6

presence. Zemplèn [1912, p. 233] on the contrary found that powdered seed kept in a vacuum desiccator over sulphuric acid for six weeks was just as active as the fresh material. The powdered seed kept by me, however, was simply corked up in a test tube.

Summary.

(1) The urease activity of Sword bean (*Canavalia ensiformis*) is many times greater than that of any of the Soy bean varieties which were tested. This is the case with both American and Indian seed. The table shows that an extremely rapid production of ammonium carbonate takes place immediately on adding sword bean extract to urea solution.

(2) The urease activity of five of the six varieties of soya bean examined is practically identical. The sixth variety is distinctly more active.

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L. ON THE SO-CALLED "ACETONE-SOLUBLE PHOSPHATIDES."

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(*Received August 20th, 1914.*)

Though many points relating to the nature and distribution of the phosphatides still await solution, it has recently been shown that certain bodies which were supposed to be definite substances are really nothing more than lecithin contaminated by nitrogenous impurities [MacLean, 1913]. This observation has materially simplified the phosphatide problem, and it seems probable that further research will reduce the number of these bodies to a comparatively few well-defined substances. It is now definitely established that the only alcohol-soluble and acetone-insoluble phosphatide present in the majority of tissues is lecithin. The substance described as a di-amino-monophosphatide, and obtained from ether-extracted tissues by subsequent extraction with alcohol, is not a definite substance but a mixture of lecithin with certain basic bodies. Lecithin can be easily obtained from the mixture by emulsification with water and precipitation with acetone [MacLean, 1912]. The only tissue phosphatides identified with certainty are lecithin, kephalin, cuorin and sphingomyelin, and all these substances differ from ordinary neutral fat and from fatty acids in the fact that they are insoluble in acetone.

Under certain conditions lecithin is precipitated almost quantitatively from its ethereal solution by excess of acetone, and this property is constantly made use of in the separation of the phosphatides from accompanying fats and fatty derivatives.

While it is agreed that phosphatides in general are insoluble in acetone, certain observers have described another distinct class of these bodies—*acetone-soluble phosphatides.*

Thus Fränkel and Pari [1909] extracted ox-pancreas with acetone and from the acetone extract obtained a phosphatide which was isolated as the cadmium chloride combination. This compound, which they called vesalthin, had a nitrogen to phosphorus ratio of 1 : 1 and in many ways did not materially differ from the compound formed by lecithin with cadmium chloride. It had an empirical formula represented by $C_{32}H_{63}O_9NP \,.\, CdCl_2$ and contained

$$1{\cdot}71\ \%\ N \text{ and } 3{\cdot}78\ \%\ P. \quad N : P = 1 : 1.$$

Erlandsen [1907] obtained a somewhat similar substance from ox-heart; this substance he isolated as the platinum derivative. The dried heart tissue was extracted with ether, the ethereal extract concentrated to a syrup and excess of acetone added; on separating the precipitated lecithin the acetone solution still contained a phosphatide together with other acetone-soluble fatty bodies. On concentration of the acetone solution the phosphatide was not precipitated by the addition of more acetone but easily passed into solution. The residue obtained on evaporation of the acetone was extracted with a little alcohol and the phosphatide precipitated by the addition of an alcoholic solution of platinum chloride. The precipitate was washed with alcohol and dried in vacuo. The platinum chloride combination thus obtained had the empirical formula $(C_{33}H_{62}O_8NP)_2H_2PtCl_6$;

$$N = 1{\cdot}81\ \%, \quad P = 3{\cdot}69\ \%; \quad N : P = 1{\cdot}09 : 1.$$

Now since lecithin has approximately the formula $C_{43}H_{80}O_9NP$ with 1·8% nitrogen and 4% phosphorus, it is obvious that the phosphatides of the above compounds of Fränkel and of Erlandsen contain a good deal more nitrogen than is present in lecithin. In fact the nitrogen percentage of the above *compounds* is almost exactly the same as that of *free* lecithin, while lecithin-cadmium-chloride contains about 1·4 % nitrogen and 3·90 % phosphorus.

Since it has been shown that lecithin very often contains impurities of a basic nature together with phosphorus compounds, it was possible that the above acetone-soluble phosphatides were nothing but impure lecithin which, owing to some physical or chemical change, had become soluble in acetone.

In order to test this, an ethereal extract of dried ox-hearts was made in the usual way. The greater part of the ether was evaporated, the lecithin precipitated by the addition of a large excess of acetone and the acetone filtrate evaporated to a thick syrup. This syrup contained large amounts of nitrogen and phosphorus indicating the probable presence of a phosphatide, but on the addition of more dry acetone no precipitate whatever was obtained.

This acetone was evaporated under reduced pressure and the syrup obtained allowed to flow slowly drop by drop into a large bulk of acetone in a tall cylinder. For each cc. of syrup about 200 cc. of acetone were taken. Immediately on the addition of the first few drops the liquid became hazy and then opalescent. After adding the calculated amount of syrup, the cylinder was shaken thoroughly, when a well-marked bulky flocculent precipitate separated out, leaving the yellowish tinged acetone quite clear. After standing overnight the supernatant fluid was separated, the acetone again evaporated and the syrup tested for nitrogen and phosphorus. As both were still present in considerable amount, the syrup was again added drop by drop to excess of acetone. Again a slight haziness appeared, followed by the formation of a small amount of flocculent precipitate. A third repetition of this process gave a very slight precipitate, but on the fourth attempt, the acetone to which the syrup was added remained perfectly clear, not a trace of precipitate being produced. On evaporation of the acetone the residue still gave distinct reactions for nitrogen and phosphorus. For purposes of description later on this residue will be called *syrup M*.

The different precipitates obtained above were collected and dissolved in a small amount of ether. On addition of acetone a well-marked precipitation at once took place and the substance behaved in all respects like lecithin. On analysis, however, the nitrogen content was found to be much too high, so the syrup was purified by emulsification with water and precipitation with acetone [MacLean, 1912]. A considerable amount of some basic impurity together with some phosphorus was separated off. The purified phosphatide was then dissolved in ether, precipitated with acetone and dried in vacuo over H_2SO_4. Analysis gave

$$N = 1{\cdot}81\ \%;\quad P = 3{\cdot}96\ \%;\quad N : P = 1{\cdot}01 : 1.$$

It was soluble in ether, alcohol, chloroform and benzene but quite insoluble in acetone. Its alcoholic solution was precipitated by cadmium chloride and by platinum chloride. On hydrolysis, choline was obtained. Its behaviour, in all respects like ordinary lecithin, left no doubt that this "acetone-soluble" substance was really lecithin.

An attempt was now made to investigate the substance present in the acetone syrup M mentioned above. When added drop by drop to pure dry acetone not the faintest haze appeared. Since it is well known that the presence of electrolytes often plays an important part in the precipitation of lecithin, it was thought that the addition of a trace of electrolyte to the

acetone might give a precipitate. Since the acetone used in these experiments was carefully dried over calcium chloride and distilled immediately before use, no electrolyte could be present. A trace of calcium chloride was now added to the acetone and the syrup run in drop by drop as before. Immediately a marked opalescence appeared, followed by a very distinct precipitate. This process was repeated as described above until no more precipitate could be obtained. The final acetone solution, on evaporation to a syrup, was found to be *free from nitrogen and contained only a very faint trace of phosphorus*. Thus the whole of the phosphatide was precipitated by acetone in the presence of a minute quantity of an electrolyte.

The different precipitates were mixed together, dissolved in ether, filtered and precipitated by acetone. The precipitate obtained was purified in the usual way with water and acetone, dissolved in ether, again precipitated with acetone and dried in vacuo. Analysis gave:

$$N = 1{\cdot}86\ \%;\quad P = 3{\cdot}98\ \%;\quad N : P = 1{\cdot}03 : 1.$$

In solubilities and other physical and chemical properties, it behaved exactly as ordinary lecithin. The whole of the phosphatide present in the original solution was therefore lecithin.

In another experiment, two ox-hearts were extracted with alcohol, the alcohol evaporated, the residue taken up with ether and precipitated with acetone as described. The acetone solution was allowed to stand in the ice-chest over night when a slight precipitate formed. This was filtered off, the acetone evaporated and the residue added drop by drop to excess of acetone containing *a trace of calcium chloride*. The proportions taken were 4 cc. syrup to 100 cc. acetone. A very marked flocculent precipitate was obtained. On separating the precipitate, evaporating the acetone and repeating the above process, another well-marked precipitate separated. On the other hand when a small amount of the syrup was added to pure acetone free from electrolytes the fluid remained quite clear.

The process was repeated six times, when it was found that practically no substance separated on the addition of the syrup to the acetone containing calcium chloride.

The syrup now obtained on the evaporation of the acetone contained 0·025 % phosphorus and 0·362 % nitrogen, *i.e.*, N : P = 11 : 1. The original syrup was divided now into parts:

A	*B*
Residual syrup which gave no precipitate when added to excess of acetone containing an electrolyte.	Precipitate obtained by adding syrup to acetone containing an electrolyte.

Syrup A. From the relation of the nitrogen to phosphorus it was obvious that only a small portion of the nitrogen, if any, was present in phosphatide form. On addition of an alcoholic solution of cadmium chloride no precipitate was obtained unless excess was added, when a slight opalescence occurred. It cleared up immediately on the addition of excess of alcohol. The same phenomenon occurred when platinum chloride in alcoholic solution was added.

When three drops of a 1 % alcoholic solution of lecithin were mixed with 5 cc. of the syrup, the addition of a few drops of cadmium chloride gave a distinct precipitate which did not disappear in excess of alcohol. Thus when a trace of lecithin was added the behaviour of the syrup was entirely different from that of the control, showing that the substance originally present was not lecithin. The reactions of the syrup with cadmium chloride and platinum chloride were exactly the same as those obtained with a basic water-soluble impurity which I had previously shown to be constantly associated with tissue lecithin [MacLean, 1913].

On extracting the syrup three times with water, the aqueous solution contained all but a trace of the nitrogen of the syrup, while no phosphorus could be detected. The part insoluble in water gave no precipitate whatever on the addition of cadmium chloride or platinum chloride in excess and was practically free from nitrogen. It is, therefore, clear that the nitrogen of the syrup was present in some water-soluble combination which contained no phosphorus or at most only a trace of phosphorus. This entirely excludes phosphatides.

Precipitate B obtained by adding syrup to acetone containing an electrolyte.

This substance was dissolved in ether and precipitated with acetone. It was then purified three times with acetone and water, and a water-insoluble residue obtained. The acetone-water solutions were mixed together and evaporated to a syrup. Substance *B* was thus divided into two parts:

(1) A portion insoluble in water.

(2) A water-soluble portion.

(1) *The water-insoluble portion.*

This constituted the greater part of the substance *B*. It was soluble in alcohol, ether, chloroform and benzene. From its ethereal solution it was precipitated almost quantitatively by acetone even in small amount,

It contained choline and its physical characteristics appeared identical with those of lecithin. On analysis it gave:

$$N = 1{\cdot}85\ \%;\quad P = 4{\cdot}05\ \%;\quad N : P = 1{\cdot}01 : 1.$$

From its analyses and reactions there can be no doubt that the substance was ordinary lecithin.

(2) *The water-soluble portion.*

This had all the properties of the water-soluble substance first described. It was insoluble in ether, soluble in alcohol containing a trace of moisture, but practically insoluble in water-free alcohol. On hydrolysis, no fatty acids were produced. Analysis gave:

$$N = 2{\cdot}05\ \%;\quad P = 0{\cdot}43\ \%;\quad N : P = 10 : 1.$$

A third experiment carried out with an old extract of heart on the lines indicated gave similar results. A substance was obtained from the acetone extract which had all the reactions of lecithin. Analysis gave:

$$N = 1{\cdot}89\ \%;\quad P = 3{\cdot}94\ \%;\quad N : P = 1{\cdot}05 : 1.$$

From these results it is clear that the 'acetone-soluble' phosphatide described as obtained from ox-heart is really ordinary lecithin associated with a nitrogenous impurity. This lecithin is easily precipitated by acetone when certain electrolytes are present, but in the absence of these electrolytes the presence of fatty acids and fats renders it soluble in acetone. When separated, it behaves in all respects like ordinary lecithin both towards acetone and other reagents.

It is very probable that all phosphatides are insoluble in acetone, and that an application of the above methods to other tissues would result in eliminating from the literature certain substances which are supposed to be individual phosphatides, but which are really nothing more than impure lecithin.

SUMMARY.

(1) The so-called acetone-soluble phosphatide of heart is impure lecithin. This substance can be easily separated from accompanying fats and fatty acids by means of acetone *containing a small amount of some electrolyte, such as calcium chloride.* In this condition it is associated with a basic impurity. When purified it has all the reactions of lecithin and is quite insoluble in acetone.

(2) The basic impurity is not a phosphatide; it is soluble in water and contains no fatty acids, while only a small and variable amount of phosphorus is present.

(3) It is probable that all acetone-soluble phosphatides described in the literature are merely lecithin contaminated with the nitrogenous impurity mentioned.

Part of the cost of this research has been defrayed by a Government grant from the Royal Society.

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LI. A NOTE ON THE BLACK PIGMENT IN THE SKIN OF AN AUSTRALIAN BLACK.

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(*Received August 25th, 1914.*)

The pigment occurring in the skin of black races is stated to absorb the violet and ultraviolet rays in the solar spectrum, and thus to act as a protection against the harmful effects of tropical sunlight. This absorption has only been demonstrated in the case of the whole skin [1913], and no references are to be found in the text books on Biochemistry to any work on the absorption spectrum of any of the melanin pigments which have been prepared from various sources.

The present note contains an account of the preparation of melanin, from the skin of an Australian Black who died in the local hospital, which was prepared in order to study the absorptive action on light of the isolated pigment.

The only instance recorded in the literature of the isolation of melanin from human skin is that of Abel and Davis [1896], who prepared a black pigment from the skin of a negro. The tissue was destroyed by heating either with 5–6 % potassium hydroxide, or with concentrated hydrochloric acid, and the pigment separated in the form of granules which contained the pigment and the pigment structure. The melanin was set free from these "pigment granules" by treatment for several days with hydrochloric acid. It was then extracted with a warm solution of potassium hydroxide in which it slowly dissolved, and finally precipitated by the addition of alcohol and ether. It was purified by repeatedly dissolving in warm alkali and reprecipitating. Abel and Davis found the composition of the "pigment granules" to be:

C = 52·83 %, H = 3·86 %, N = 14·01 %, S = 3·6 %, Fe = – %

and of the free pigment after treatment with alkali:

C = 53·56 %, H = 5·11 %, N = 15·47 %, S = 2·53 %.

In a paper on the melanin in black wool, Gortner [1910] stated that this pigment was readily destroyed by continued heating with a solution of caustic alkali. He recommended for the extraction the use of hot very dilute (0·2 per cent.) sodium hydroxide which dissolved out the pigment without decomposing it. In his opinion the heating with strong alkalies or acids, employed by Abel and Davis and by other workers in the extraction of melanins from other sources, was calculated to decompose a large proportion of the pigment.

In the present instance, however, it was found that a solution of alkali of the strength recommended by Gortner was too dilute to attack the skin, and in the preparation described below 5 per cent. potassium hydroxide was employed and the melanin dissolved out by heating on the water bath with this solution. To avoid continued heating of the dissolved melanin with the alkali, the material was treated with a successive number of small quantities of the solution, each portion being only allowed to act for a short time. The melanin when purified had the composition :

C = 60·12 %, H = 6·70 %, N = 11·89 %, S = — %, Fe = 0·21 %,

thus differing from that obtained by Abel and Davis. On combustion in oxygen a small quantity of ash was left, which contained iron. Abel and Davis record the presence of iron only in the "pigment granules."

A solution of the pigment both in 5 per cent. potassium hydroxide and in concentrated sulphuric acid was examined spectroscopically. In both cases the solutions absorbed all the rays in the violet, the blue and in part of the green to a wave length of about 515μ. Beyond this point there was slight blurring of the green and orange, whilst the red was practically unaffected.

Experimental.

A piece of skin from the back, 450 grams, was cut up as finely as possible and was warmed on the boiling water bath with 400 cc. of a 5 per cent. solution of potassium hydroxide. After about half an hour the solution was filtered through glass wool, and most of the liquid squeezed out of the residue by pressing with a pestle. The residue was then treated with a fresh lot of alkali in the same manner, and this process repeated until the material had been thoroughly macerated. The bulk of the material including the pigment went gradually into solution, and the filtrates on cooling deposited a good deal of fat which could be removed by filtering through glass wool. Each lot of extract as obtained, was at once cooled, most of the fat removed,

and the mixture acidified with hydrochloric acid. The pigment was thereby precipitated as a dark brown powder, and was gradually collected by filtering each of the mixtures through the same paper.

When as much as possible had been obtained, it was purified by repeatedly dissolving in warm 5 per cent. potassium hydroxide, filtering and precipitating with hydrochloric acid. The last traces of acid were removed by repeatedly suspending in distilled water and filtering. The brown powder was then dried on the filter paper by washing with alcohol and ether, and the paper folded and extracted in a Soxhlet apparatus, first with alcohol, and then with ether. Finally it was washed with pure carbon bisulphide, then with pure ether, and dried in a vacuum over sulphuric acid.

The product was a dark brown powder, insoluble in water. It was readily soluble in warm dilute potassium hydroxide forming a brown solution. It dissolved in cold concentrated ammonia and in cold concentrated sulphuric acid, being precipitated from the latter by dilution with water.

The substance contained carbon, nitrogen, sulphur and a trace of iron.

When dried it was found to take up moisture very rapidly, so that the samples taken for analysis were dried to constant weight in a vacuum over phosphoric anhydride at the temperature of boiling toluene. After combustion in oxygen a very small quantity (0·5 %) of a red coloured ash was left, in which the iron was determined colorimetrically with potassium thiocyanate, a standard iron solution being employed for comparison.

The nitrogen was determined by Kjeldahl, but unfortunately the quantity of material available was extremely small, so that the result must be accepted with some reservation.

Sufficient material was not obtained for a quantitative analysis of the sulphur. Analysis :

0·1169 g. ; 0·2577 g. CO_2 ; 0·0700 g. H_2O ; 0·25 mgm. Fe.
0·0387 g. ; 0·0046 g. N.
C = 60·12 % ; H = 6·70 % ; N = 11·89 % ; Fe = 0·21 %.

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LII. QUANTITATIVE ESTIMATION OF ASPARTIC AND GLUTAMINIC ACIDS IN THE PRODUCTS OF PROTEIN HYDROLYSIS.

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(*Received July 28th, 1914.*)

The figures obtained by the majority of workers when estimating glutaminic acid in caseinogen by separating the hydrochloride in the usual way, are uniformly about 11 per cent. In two cases, however, very much higher yields of the recrystallised hydrochloride have resulted. Thus Osborne and Guest [1911] found 15·55 per cent., and Foreman 15·9 per cent. (unpublished result). In these two cases the most favourable conditions for the separation were obtained, but could not be clearly defined. The accuracy of the results, however, still remained in doubt, and the method is unreliable.

The ease with which the glutaminic acid hydrochloride separates seems to vary with the protein under treatment and with the amount of glutaminic acid present. Plimmer [1912] states that separation of the hydrochloride "occurs in the case of caseinogen and certain vegetable proteins which contain from 10–40 per cent. of this amino-acid." If the protein contains under 10 per cent., then, as a rule, no hydrochloride will separate. Occasionally, however, a separation is obtained; thus Hopkins and Savory [1911] working with Bence-Jones protein obtained 8·05 per cent.

When a protein contains so little glutaminic acid that separation as the hydrochloride before esterification is impossible, or any other difficulties are encountered, the estimation of this amino-acid must depend upon the working up of the higher boiling esters. The yield of glutaminic acid hydrochloride obtained in this way is always very poor when compared with that obtained by separation before esterification. An investigation of the unesterified portion will show that glutaminic and aspartic acids, probably

owing to their dibasic character, do not esterify so readily. From a quantitative standpoint, therefore, at any rate, the percentage of glutaminic acid obtained by working up the higher boiling esters appears to be valueless.

A direct method for the estimation of these two amino-acids is described below.

It seemed possible that a method might be based on some essential difference between the two types—dibasic and monobasic amino-acids. As it is well known that the calcium salts of non-nitrogenous dibasic organic acids have a much higher degree of insolubility in water or alcohol than the calcium salts of non-nitrogenous monobasic acids, it was thought that the same principle might apply in the case of the dibasic and monobasic amino-acids. Experiments were therefore made with a view to testing this matter.

Calcium chloride solution was added to a solution of the amino-acids obtained by the hydrolysis of caseinogen. No precipitate resulted, and on adding much alcohol only a small precipitate was obtained. When, however, another portion of the same solution was made alkaline with lime, a copious precipitate appeared on the addition of alcohol. The precipitate seemed to increase somewhat in quantity when the free ammonia was removed before the alcohol was added.

Solutions of all the monamino-acids found in proteins, with the exception of oxyproline, were then treated separately with lime and alcohol. Glutaminic and aspartic acids, cystine and tyrosine all gave calcium salts insoluble in alcohol. The calcium salts of the other monamino-acids, however, were found to be very soluble in alcohol, and the solutions all remained perfectly clear when the alcohol was added. Pyrrolidonecarboxylic acid has since been tried in the same way, and a copious precipitate was obtained.

Since these observations were made I have found that Abderhalden and Kautzsch [1910] have made the calcium salts of aspartic, glutaminic, and pyrrolidonecarboxylic acids separately from the pure substances in a similar way. They have not suggested, however, that any quantitative use could be made of these facts in reference to separations from hydrolytic products derived from proteins.

The precipitate obtained, by means of lime and alcohol, from the products of the hydrochloric acid hydrolysis of caseinogen, has been found to consist of the calcium compounds of the following substances:

(*a*) glutaminic and aspartic acids;

(*b*) a gummy substance very difficult to crystallise;

(*c*) a very small quantity of a pigmented substance precipitated from the cold aqueous solution by silver sulphate solution;

(*d*) a substance precipitated by phosphotungstic acid.

The calcium salt of tyrosine separates from the alcoholic filtrate on standing a long time.

The phosphotungstic precipitate, derived in the manner stated, from 20 g. caseinogen gave 0·57 g. dry substance after removing the phosphotungstic acid. An aqueous solution of this substance was neutralised with nitric acid and silver nitrate solution added. No precipitate resulted. On adding ammonia, however, a precipitate was obtained. This dissolved in excess. The further investigation of this substance is postponed.

Description of the Method.

20–40 g. of the protein are hydrolysed by boiling for 48 hours with three times the quantity of hydrochloric acid, and the liquid evaporated to a syrup at 45° and 15 mm., thus removing the large excess of hydrochloric acid.

Formation of the calcium salts and removal of the humins. The syrup is taken up in 200–400 cc. water, depending upon the quantity of protein taken. Pure calcium oxide is slaked, made into a cream with water, thoroughly cooled, and added to the liquid in sufficient quantity to ensure a good excess. As a rule, 0·5 g. calcium oxide to every gram of protein appears to be required. The flask is corked, and the cold liquid shaken for a few minutes and then filtered on a Buchner funnel, and the residue thoroughly washed with cold water. The insoluble part contains the humin bodies and the excess of lime.

Removal of excess of water and ammonia. The slightly coloured filtrate is evaporated at 40–45° and 15 mm., so that the remaining liquid will possess a volume corresponding to 3·5–4 cc. for each gram of protein taken. This operation also serves to remove all traces of ammonia which can be estimated if desired. As I have shown elsewhere [Foreman, 1914] glutaminic acid is converted into pyrrolidonecarboxylic acid to a considerable extent when its solution is boiled with excess of lime. No such change, however, occurs at 40–45°. The temperature is therefore not allowed to exceed this limit. The tube admitting air into the distilling flask should be fitted with a soda lime tube to prevent the entrance of carbon-dioxide.

Precipitation of the calcium salts insoluble in alcohol. Rectified spirit (97 per cent. alcohol) is now added to the solution in the distilling flask in small quantities at a time with vigorous shaking. The alcohol should be

added gradually, in order to prevent the precipitate forming a clot which would tend to carry down other substances. The first part of the precipitate is inclined to be sticky, and if the alcohol be gradually added with shaking, this part adheres to the sides of the flask as a thin uniform layer which can be afterwards easily washed. The addition of the alcohol is continued until further precipitation ceases. About a litre of alcohol is required as a rule.

The precipitate is now filtered on a Buchner funnel and the flask with its adhering layer as well as the contents of the funnel washed thoroughly with rectified spirit. Occasionally when the alcoholic washings enter the clear main filtrate, an opalescence appears on standing, and an insignificant amount of precipitate may separate. When this is filtered off further similar separations may be obtained in a like manner. These small precipitates contain no glutaminic or aspartic acids and may be neglected. The calcium salt of tyrosine separates slowly from such a mixture in this manner.

Quantitative removal of the calcium from the alcohol-insoluble calcium salts. The precipitate is taken up in about 300 cc. water and the calcium quantitatively removed by means of oxalic acid.

Removal of traces of chloride and a pigmented substance precipitated by cold silver sulphate solution. The calcium oxalate is filtered off and aqueous silver sulphate solution added to the cooled liquid until no further precipitate results. This removes a small amount of chloride and also a very small quantity of a peculiar pigmented substance, the precipitate presenting a flocculent appearance. If the liquid be heated on the water bath before filtering, the silver compound of the pigmented substance dissolves but the substance is precipitated by phosphotungstic acid, and is included in the precipitate obtained later on.

The slight excess of silver is now removed from the solution by means of sulphuretted hydrogen.

The slightly coloured filtrate from the silver sulphide is reduced to about half its bulk on the water bath to remove sulphuretted hydrogen.

Removal of substance precipitated by phosphotungstic acid. An aqueous solution of phosphotungstic acid (Merck's) is then added until the colour disappears from the solution, 5–10 g. being usually sufficient. The liquid at this stage will continue to yield insignificant traces of precipitate if more of the phosphotungstic acid solution be added, and about as much again is required in order to carry the precipitation to absolute completion. This further addition of phosphotungstic acid is unnecessary, however, as the following test shows.

A small test portion of the liquid is taken after the colour has disappeared, and made up so as to contain 5 per cent. sulphuric acid. To this is added a few drops of a 25 per cent. solution of phosphotungstic acid in 5 per cent. sulphuric acid. No precipitate results. By adding no more phosphotungstic acid after the disappearance of the colour, one avoids a much larger precipitate of barium phosphotungstate to be obtained on removing the excess, and possible loss of the amino-acids by occlusion is thus reduced to a minimum. The usual method of precipitation in 5 per cent. sulphuric acid was avoided, as in this case the presence of the acid is unnecessary, and loss by occlusion in a large barium sulphate precipitate, produced on its removal, is prevented.

Baryta water is now added to the solution at 45° until no further precipitate results. If the solution at this stage possesses a blue colour[1], the addition of baryta water should be continued until the colour completely disappears.

The small precipitate of barium phosphotungstate is filtered off, and the slight excess of baryta quantitatively removed by means of sulphuric acid.

Separation of the monamino-acids in the dry state. The small barium sulphate precipitate is filtered off, and the liquid evaporated to about 50 cc. on the water bath. It is then transferred to a weighed dish, containing a weighed stirring rod, and slowly evaporated to 20–25 cc. (corresponding to 40 g. protein taken originally) on a warm water bath. No trace of tyrosine appears. The amino-acids crystallise on cooling, and the semi-solid mass is stirred and placed in a vacuum desiccator over calcium chloride over night. By the next morning the amino-acids will have dried to hard gritty cakes. These can be broken up with the rod and placed for a further period in the vacuum desiccator, but as a rule the loss of weight in this second period is very small indeed.

Separation of the glutaminic and aspartic acids by extraction with cold glacial acetic acid in which these acids remain undissolved. The dry substance is then triturated with cold glacial acetic acid in the dish by means of a small glass pestle, and filtered on a small Buchner funnel. The glutaminic and aspartic acids remain undissolved. The solid is again placed in the dish and extracted in the same way with more cold glacial acetic acid. It is finally all transferred to the filter and washed two or three times with glacial acetic acid. The white solid, which comes off the filter paper quite cleanly, is transferred to a weighed dish, dried and weighed.

[1] A blue colouration appeared in the filtrate when using Kahlbaum's phosphotungstic acid. This did not occur when Merck's product was employed.

Treatment of the acetic acid extract. The extract yields a gum on removing the acetic acid. It is placed in a weighed porcelain dish and the acetic acid removed as completely as possible *in vacuo* at 40–45°.

The remaining traces of acetic acid can be removed in a vacuum desiccator over strong potash. Further drying may be carried out *in vacuo* over calcium chloride if considered necessary.

A further trace, usually under 0·1 g., may be obtained by extracting this dry residue a second time.

Estimation of pyrrolidonecarboxylic acid, present in the substance soluble in cold glacial acetic acid.

The moist gum is now weighed quickly and thoroughly mixed by stirring with a strong glass rod. The rod is then used to transfer a 0·2 g. portion to a weighing tube for an amino-nitrogen determination. Without delay a further 0·5 g. portion is weighed into a bulb of 4–5 cc. capacity, and about 2 cc. strong hydrochloric acid added. A long tube is then sealed to the stem of the bulb to act as a reflux condenser. The bulb is allowed to rest lightly on a sand bath, and the liquid boiled for about 12 hours. The liquid is then made up to 20 cc. with water, and the amino-nitrogen determined in a portion of this solution. The difference between the amino-nitrogen present before and after boiling with hydrochloric acid represents pyrrolidonecarboxylic acid. The total amount of this acid present in the moist gum can then be found, and its equivalent of glutaminic acid calculated. This is added to the weight of glutaminic acid separated as such, before calculating the percentage in the protein.

If confirmation be required, the remainder of the moist gum may be extracted with cold absolute alcohol in which pyrrolidonecarboxylic acid is soluble. The alcohol can then be evaporated as completely as possible from this extract in a weighed dish and the weight of this alcohol-soluble substance obtained. The amino-nitrogen can then be determined in portions weighed out, without delay, before and after boiling with strong hydrochloric acid in the manner already described.

Instead of weighing out portions of the gum it would have been easier to have made its aqueous solution up to a known volume. This procedure, however, was not adopted because of the difficulty of transferring the gum, derived from the evaporation of an aliquot portion, to the bulb for boiling with hydrochloric acid.

Estimation of the proportion of glutaminic acid to aspartic acid in the mixture of these two acids obtained.

The white substance, which was insoluble in cold glacial acetic acid, is then submitted to analysis. The proportion of glutaminic acid to aspartic acid is calculated on the carbon content of the mixture.

As the differences between the percentages of carbon in glutaminic and aspartic acids is as great as 4·73 the proportion can be determined with tolerable accuracy.

The purity of the mixture, and the proportions calculated from the carbon content can be checked by a nitrogen determination.

Separation of the two constituents of the mixture for further identification.

A known weight of the mixture is converted into the copper salts in plenty of water, and the filtered liquid allowed to cool. If crystals of copper aspartate do not separate after standing some time, the liquid is gently reduced in bulk until signs of crystallisation appear. After standing over night the light blue needles are filtered off, and an attempt may be made to obtain a further crop from the mother liquor in the same way. As a general rule, however, the first crop will contain practically all the aspartic acid. At any rate, this has been shown to be true when the aspartic acid is present to a greater extent in the mixture than the glutaminic acid. Occasionally, if allowed to stand too long, the characteristic small heavy dark blue prisms of copper glutaminate may appear mixed with the light blue needles. These crystals, however, can be separated quite easily from the needles by elutriation with cold water.

The filtrate from the copper aspartate is freed from copper by means of hydrogen sulphide and the liquid evaporated to dryness in a weighed dish. After taking the weight of the dry substance hydrochloric acid is added in the proportion of 1·8 to 2 cc. for each g. substance. The liquid is boiled for a short time, cooled and saturated with dry hydrochloric acid gas in the usual way. The glutaminic acid hydrochloride thus obtained can be weighed and analysed.

A maximum separation of glutaminic acid hydrochloride appears to result when the substance and the hydrochloric acid are taken in the proportion stated.

Should copper aspartate refuse to separate through being present in the mixture in too small a proportion, the glutaminic acid must be separated first as the hydrochloride. The filtrate from this is then evaporated to dryness and the copper aspartate may be obtained by working up this residue.

Results Obtained by Applying the Method to the Products of the Hydrolysis of Caseinogen.

The method as above described was applied to 40 g. "Hammarsten casein" containing 14·08 per cent. nitrogen and 9·3 per cent. moisture. Calculating on the basis that pure caseinogen contains 15·62 per cent. nitrogen, the 40 g. is equal to 36·05 g. of the pure substance. The hydrolysis was carried out by boiling with three times the quantity of hydrochloric acid (sp. gr. 1·16) for 48 hours. The following results were obtained:

Total weight of dry substance before extraction with the cold glacial acetic acid			13·48 g.
Weight of glutaminic and aspartic acids insoluble in the cold glacial acetic acid, dried to constant weight at 100°			
The first extraction	6·996 g.		
The second extraction	·025 g.		7·021 g.
Difference = weight of dry gum			6·46 g.

Analysis of the mixture of glutaminic and aspartic acids.

The white substance weighing 7·02 g. dried to constant weight at 100° gave the following results on analysis:

0·1684 g.; 0·2494 g. CO_2; 0·0902 g. H_2O.

0·1550 g.; 12·8 cc. nitrogen at 20° and 768 mm. by Dumas' method.

0·1634 g.; 27·0 cc. amino-nitrogen at 20° and 768 mm. by van Slyke's method.

The percentages calculated from these results are shown below side by side with the theoretical percentages present in glutaminic and aspartic acids:

	Calculated for glutaminic acid	Found	Calculated for aspartic acid
Carbon	40·82	40·39	36·09
Hydrogen	6·12	5·95	5·26
Nitrogen	9·52	9·57	10·52
Amino-nitrogen ..	9·52	9·56	10·52

The proportion of aspartic acid to glutaminic acid in the mixture is therefore shown as follows:

$$\frac{40{\cdot}82 - 40{\cdot}39}{40{\cdot}82 - 36{\cdot}09} = \frac{1}{11};$$

$$\frac{1}{11} \text{ of } 7{\cdot}02 = 0{\cdot}64 \text{ g. aspartic acid,}$$

$$\frac{10}{11} \text{ of } 7{\cdot}02 = 6{\cdot}38 \text{ g. glutaminic acid.}$$

Estimation of pyrrolidonecarboxylic acid in the gummy substance soluble in the cold glacial acetic acid.

Practically the whole of the 0·025 g. substance obtained by the second extraction came from the acetic washings of the 7 g. Through determining this fact two lots of gum were obtained :

A. from the main filtrate ;
B. from the washings.

These were treated separately, as it was afterwards found inconvenient to mix them.

When dried, as far as possible in the manner described, the two portions of the moist gum weighed as follows :

A. 6·29 g.
B. 2·48 g. } equal to 6·46 g. in the dry state.

The amino-nitrogen was then determined before and after boiling for 16 hours with hydrochloric acid, with the following results :

A. 0·161 g. gave 13·8 cc. amino-nitrogen at 20·8° and 768 mm. 0·4542 g. was boiled with 2·5 cc. strong hydrochloric acid for the time stated, and then made up to 20 cc. 10 cc. of this gave 27·1 cc. amino-nitrogen at 24·3° and 762 mm.

B. 0·2100 g. gave 19·2 cc. amino-nitrogen at 19° and 771 mm. 0·4705 g. was treated with hydrochloric acid in exactly the same way as *A*, and then made up to 20 cc. 8·6 cc. of this gave 22·9 cc. amino-nitrogen at 23° and 763 mm.

From these figures the following results were calculated :

Weight of moist gum	Percentage of amino-nitrogen before boiling with HCl	Percentage after boiling with HCl	Difference
A. 6·29 g.	4·94	6·70	1·76
B. 2·48 „	5·24	6·41	1·17

Calculating the increase in amino-nitrogen into terms of glutaminic acid, it will be found that 1·163 g. is thus obtained from *A* and 0·305 g. from *B*, making a total of 1·468 g.

In order to prove that this increase in amino-nitrogen was due to the hydrolysis of pyrrolidonecarboxylic acid, the remaining gummy substance was extracted with cold absolute alcohol, which dissolves this acid, and the extract was boiled for 10 hours with hydrochloric acid after removing the alcohol. The brown solution, now containing glutaminic acid, was warmed

with animal charcoal and filtered. The filtrate was then evaporated to about 5 cc. and saturated with hydrochloric acid gas at 0°. The perfectly white glutaminic acid hydrochloride which separated, was then obtained in the dry state in the ordinary way, and weighed. After allowing for the portions of the original gummy substance previously removed 0·993 g. of the hydrochloride resulted.

As stated elsewhere, it is very probable that a small quantity of the pyrrolidonecarboxylic acid is decomposed when boiled with acid, thus explaining the brown colour which invariably appears. Other slight errors were introduced in the alcoholic extraction and the animal charcoal treatment. Taking these facts into consideration, the yield is consistent with that usually obtained by the hydrochloride method.

The glutaminic acid hydrochloride melted at 201–202°.

The following results were obtained on analysis:

0·1481 g.; 0·1593 g. CO_2; 0·0675 g. H_2O.

0·1529 g. gave by Dumas' method 9·1 cc. nitrogen at 18·5° and 753 mm.

0·1009 g.; 0·0091 g., equal to 9·02 per cent. of ash.

Allowing for the ash, the following figures were calculated:

	Found per cent.	Calculated for glutaminic acid hydrochloride per cent.
Carbon	32·25	32·69
Hydrogen	5·56	5·45
Nitrogen	7·52	7·62

It is difficult to account for the ash unless one assumes that it was derived from the phosphotungstic acid used early in the method.

The products of the hydrolysis of the 36·05 g. pure caseinogen therefore contained:

1·468 + 6·38 = 7·848 g. glutaminic acid,
0·64 „ aspartic acid.

The percentages in the protein work out as follows:

21·77 per cent. glutaminic acid,
1·77 „ „ aspartic acid.

Results of a previous trial with caseinogen.

For this trial, 20 g. "Hammarsten casein" equal to 18 g. of the pure substance were taken. The calcium salts were precipitated by alcohol in two fractions. Before adding the alcohol the volume was reduced at 40–45° and 15 mm. to about 100 cc. In the 40 g. trial, already described, the volume

was about 120 cc. before adding the alcohol, so that relatively more water was present when precipitating the calcium salts in this 20 g. trial.

The two fractions were obtained as follows. The alcohol was added in small quantities at a time with shaking until very little colour remained in the solution. The second fraction was obtained by completing the precipitation with more alcohol. For convenience these two lots of precipitate may be labelled *A* and *B*.

These two fractions *A* and *B* were worked up, in the way already described, omitting all precautions as to temperature. Finally the substance, instead of being dried in the desiccator before extracting with acetic acid, was heated in the dish on the water bath with occasional stirring until dry. The cakes were then crushed and dried to constant weight in the steam oven. The acetic acid was removed from the extracts on the water bath, and the gummy residues were afterwards dried to constant weight in the steam oven before analysis.

The following weights of dry substance resulted:

	Weight of dry substance before extraction with acetic acid	Weight of dry substance insoluble in acetic acid	Difference; weight of dry gum
Fraction *A*. ..	2·06 g.	1·129 g.	0·93 g.
„ *B*. ..	3·28 „	1·638 „	1·64 „
Total ..	5·34 „	2·767 „	2·57 „

Second extractions of the two gummy portions with cold glacial acetic acid yielded:

Fraction *A*. 0·081 g.
„ *B*. 0·077 „

The acetic acid was removed from these extracts on the water bath and the gums were dried in the steam oven. Portions were then taken, dried to constant weight, and analysed.

The following results were obtained:

	Fraction *A*; insoluble in acetic acid	Fraction *B*; insoluble in acetic acid	Fraction *A*. The dry gum soluble in acetic acid	Fraction *B*. The dry gum soluble in acetic acid
Carbon	40·08	40·51	45·45	46·04
Hydrogen	6·33	6·68	6·0	6·1
Amino-nitrogen ..	9·84	9·36	1·97	2·55
Kjeldahl nitrogen ..			11·26	11·31

Pyrrolidonecarboxylic acid contains:

Carbon	46·51 per cent.
Hydrogen	5·43 „ „
Nitrogen	10·85 „ „
Amino-nitrogen	nil

These two portions of gum contain smaller percentages of amino-nitrogen than was found in the gum from the 40 g. caseinogen trial. The total weight of dry substance before extraction with the acetic acid is also relatively less. Thus from the 20 g. caseinogen 5·34 g. were obtained and from the 40 g. caseinogen 13·48 g., giving a difference of 2·8 g. per 40 g. caseinogen. On calculating, it will be found that this 2·8 g. contains about 10 per cent. amino-nitrogen. By allowing less water to be present when precipitating the calcium salts with alcohol, 2·8 g. of some substance soluble in cold glacial acetic acid containing about 10 per cent. amino-nitrogen has therefore been obtained in addition. The identity of this constituent of the gum has not yet been established but the matter is receiving attention.

It will be noted that the analyses of the gums do not differ widely from the calculated figures for pyrrolidonecarboxylic acid. The total nitrogen is about 0·4 per cent. higher than the total nitrogen of this acid. If basic lead acetate solution be added to a portion of the aqueous solution of the gums a large precipitate, characteristic of pyrrolidonecarboxylic acid, results on adding plenty of alcohol. If this precipitate be filtered off and extracted with cold water, a coloured lead compound remains undissolved. On decomposing this with sulphuretted hydrogen a small quantity of brown substance (0·145 g. from 20 g. caseinogen) is obtained on evaporation. This body probably possesses a high nitrogen content, but has so far received no further attention.

In order to show more clearly that the increase in amino-nitrogen which is always obtained by boiling the gum with hydrochloric acid is due to the hydrolysis of pyrrolidonecarboxylic acid, the remaining gum was extracted with cold absolute alcohol, which dissolves pyrrolidonecarboxylic acid. The alcohol was removed from the extract, and the amino-nitrogen was determined before and after boiling with hydrochloric acid. It was argued that a much greater increase in the amino-nitrogen in this alcohol-soluble substance would give further satisfactory evidence on this point.

0·7808 g. of the dry gum was allowed to remain in contact with cold absolute alcohol for some time, in a desiccator to prevent absorption of moisture, stirring occasionally with a strong glass rod. The solution became slightly coloured, and the insoluble part gradually appeared in a powdery form. The liquid was filtered and the extraction completed with fresh cold alcohol. The alcohol was then completely removed in a vacuum desiccator over sulphuric acid, yielding 0·6267 of moist gum. Three portions of this were weighed out quickly, one for a moisture determination, and the other two

for amino-nitrogen determinations before and after boiling with strong hydrochloric acid.

0·1465 g. lost 0·0295 g. when dried at 100° till its weight was constant. The gum therefore contained 20·13 per cent. moisture, and the 0·6267 g. contained 0·5014 g. dry substance.

0·1420 g. of the moist gum gave by the nitrous acid method 4·25 cc. amino-nitrogen at 21° and 768 mm. The moist gum therefore contained 1·72 per cent. amino-nitrogen.

0·3261 g. was boiled with 1·6 cc. strong hydrochloric acid in a small bulb as before described, for 20 hours. The liquid was then made up to 20 cc. and the amino-nitrogen determined in 10 cc. of this solution. 24·2 cc. amino-nitrogen at 24·6° and 763 mm. were obtained. The percentage of amino-nitrogen calculated on the weight of moist gum taken was therefore 8·29.

By boiling with the acid the amino-nitrogen had therefore increased from 1·72 per cent. to 8·29 per cent.

Expressing this increase in terms of pyrrolidonecarboxylic acid hydrolysed by the acid, the 0·5014 g. of dry alcohol-soluble substance contained 0·3795 g. of this acid. As this 0·3795 g. was present in 0·7808 g. dry gum, 1·25 g. would be contained in the whole 2·57 g. dry gum obtained from the 18 g. pure caseinogen. This 1·25 g. corresponds to 1·42 g. glutaminic acid.

As the 0·5014 g. alcohol-soluble substance contains 0·3795 g. pyrrolidone carboxylic acid, the difference, 0·1219 g. contains the amino-nitrogen present before boiling with acid. It will be found by calculating, that this 0·1219 g. contained 8·8 per cent. amino-nitrogen. The figure should probably be nearer 10 per cent., as on boiling pure pyrrolidonecarboxylic acid with strong hydrochloric acid, 94 per cent. of change is generally accounted for, the remaining 6 per cent. probably decomposing to produce something giving the brown colour which always appears.

The portion of the 0·7808 g. gum insoluble in the alcohol amounting to 0·2704 g. and equal to 4·9 per cent. of the caseinogen should contain no amino-nitrogen. It gave a dark blue copper salt insoluble in alcohol which showed no tendency to crystallise. The investigation of this substance is proceeding.

The total weight of glutaminic and aspartic acids thus accounted for in this 20 g. caseinogen trial is shown as follows :

	Weight in g.	Percentage of the protein
Separated by means of the glacial acetic acid ..	2·925	16·22
From the pyrrolidonecarboxylic acid	1·42	7·88
	4·345	24·1

The total percentage of glutaminic and aspartic acids in the protein obtained in the 40 g. caseinogen trial was 23·54. The results obtained in the two trials therefore practically correspond.

In order to compare the two results the figures are shown side by side, as follows :

	Per cent. separated as glutaminic and aspartic acids	Per cent. glutaminic acid, estimated from the pyrrolidonecarboxylic acid content of the gum	Total per cent.
Trial *A* .. 20 g. caseinogen	16·22	7·88	24·1
Trial *B* .. 40 g. caseinogen	19·47	4·07	23·54[1]

It will be noted that in trial *A*, the amount of pyrrolidonecarboxylic acid present in the gum was greater than in trial *B*. The amount of glutaminic and aspartic acids separated as such, however, is correspondingly less. When carrying out trial *A*, however, no temperature precautions in the final stages were taken, as already explained. In another paper it will be shown that glutaminic acid changes into *l*-pyrrolidonecarboxylic acid when its aqueous solution is boiled. The change also occurs, but to a much smaller extent, at the temperature obtained in a liquid contained in an open dish on the boiling water bath. It has also been shown that even strong acids like hydrochloric and sulphuric do not completely inhibit the change until they are present to the extent of 3 per cent. and 8 per cent. respectively. These facts were not determined at the time trial *B* was carried out, the only information available at that time being that a change took place when an aqueous solution of glutaminic acid was heated with lime. The temperature precautions taken in the final stages of trial *B* were instinctive. The inference is obvious that these precautions account for the higher yield of glutaminic and the corresponding lower yield of pyrrolidonecarboxylic acid.

When carrying out trial *B* the liquid from time to time was frequently evaporated, and sometimes heated in an open dish or in a flask on the water bath, which will account for the conversion of glutaminic acid into pyrrolidonecarboxylic acid to the extent of 4·07 per cent. of the protein. As it has also been shown in another place that *l*-pyrrolidonecarboxylic acid is hydrolysed to glutaminic acid by boiling for 4 hours with strong hydrochloric acid, it is very difficult to conceive of the pyrrolidonecarboxylic acid required by this 4·07 per cent. glutaminic acid existing in the original hydrolytic liquid obtained by boiling the caseinogen for 48 hours with strong hydrochloric acid. Its formation from glutaminic acid, afterwards, appears to need no further discussion.

[1] I consider 23·54 the more accurate figure.

The Method applied to the Soluble Protein of the Swede Turnip.

This work was carried out by G. Williams, who has given me permission to refer to his results, which are awaiting publication. He was unable to obtain any glutaminic acid as the hydrochloride before esterification. In working through the ester process he obtained 2·75 per cent. aspartic and 0·26 per cent. glutaminic acid.

Using the new method he obtained 6·98 per cent. aspartic acid and 3·18 per cent. glutaminic acid, without working up the gummy substance containing pyrrolidonecarboxylic acid which it has been shown can be formed from glutaminic acid during the operations. The percentage of glutaminic acid will be increased when this gum has been worked up.

The glutaminic and aspartic acid mixture insoluble in the cold glacial acetic acid, gave the following analysis shown side by side with the calculated percentages in the pure ingredients:

	Calculated for aspartic acid per cent.	Found in the mixture per cent.	Calculated for glutaminic acid
Carbon	36·09	37·44	40·81
Hydrogen ..	5·27	5·67	6·12
Nitrogen ..	10·52	10·18	9·52

The method already described for separating the two constituents of the mixture was afterwards applied and practically all the aspartic acid was obtained as copper aspartate and nearly all the glutaminic acid partly as copper glutaminate, separated by elutriation, and the remainder as the hydrochloride from the mother liquor from the copper salts. These products all gave very satisfactory analyses.

Experiments to show that the Calcium Salts of Glutaminic and Aspartic Acids can be Precipitated Quantitatively by Means of Alcohol.

1 g. pure glutaminic acid and 1 g. pure aspartic acid were dissolved separately in about 20 cc. water, and these solutions were treated in exactly the same way. Pure calcium oxide previously made into the form of a cream with water, and cooled, was added until present in excess. The liquids were then reduced to 4 cc. at 40–45° and 15 mm. and rectified spirit added until precipitation ceased. The precipitates were filtered off and thoroughly

washed with alcohol. The filtrate and washings in each case were combined and the nitrogen content of the whole liquid determined by Kjeldahl's method. The same amount of N/10 acid, viz. 0·15 cc., was neutralised by the ammonia in each case. 99·78 per cent. of the glutaminic acid and 99·8 per cent. of the aspartic acid therefore had been precipitated under these conditions.

In a previous experiment, using 1 g. glutaminic acid, the volume of the aqueous solution of calcium glutaminate was 30–40 cc. before adding the alcohol; 83 per cent. was precipitated, determined in the way described. The amount of water present before adding the alcohol is therefore a factor that decides the yield.

It has been shown that practically the same percentages of glutaminic and aspartic acids were obtained in the 20 g. and 40 g. caseinogen trials. In the 20 g. trial, however, the volume of the aqueous solution of the calcium salts before adding the alcohol was in the proportion of 4 to 5 cc. for each gram of caseinogen originally taken, whilst in the 40 g. trial it was 3 to 3·5 cc. It therefore appears that the yield of glutaminic and aspartic acids is not affected if these limits be observed when separating them from proteins by this method. When the smaller quantity of water is present, however, more amino-nitrogen is present in the glacial acetic acid extract.

Additional evidence for the quantitative character of the precipitation of the calcium salts of the two dibasic acids was obtained by working up the alcoholic filtrate from the 40 g. caseinogen trial. The alcohol was removed, and the remaining liquid reduced to a smaller bulk than for the first precipitation. Alcohol was again added and a very small amount of precipitate, which took a long time to settle, resulted. This was filtered off, and two further small quantities obtained in succession by repeating this whole operation. These three precipitates were combined, and worked up in the same way as the main precipitate. No traces of glutaminic and aspartic acids, however, were found. The aqueous solution obtained at the finish which should have contained these acids if they had been present was neutral to litmus even when highly concentrated, and the small quantity insoluble in cold glacial acetic acid was found to consist of tyrosine. It is therefore claimed that all the glutaminic and aspartic acids were present in the main precipitate.

Summary.

1. The calcium salts of glutaminic and aspartic acids are precipitated quantitatively from their aqueous solutions by means of alcohol, provided that these solutions are sufficiently concentrated.

2. These calcium salts are also precipitated quantitatively in the same manner, from a solution containing the calcium salts of the amino-acids resulting from the acid hydrolysis of proteins.

3. Glutaminic and aspartic acids are practically insoluble in cold glacial acetic acid, and this fact is made use of in separating them from the other products obtained.

Pyrrolidonecarboxylic acid was present in the acetic acid extract.

4. Evidence is given here, as well as in another paper [1914], to show that glutaminic acid is converted into pyrrolidonecarboxylic acid to some extent during the operations, through exposing the solutions from time to time to temperatures in the neighbourhood of 80–90°.

This pyrrolidonecarboxylic acid can be reconverted into glutaminic acid by boiling with hydrochloric acid, and the degree of change from ring nitrogen to the amino-form enables an estimation to be made.

5. Other unidentified substances probably of great importance are obtained by the method.

6. The application of the method to two proteins has given substantially greater yields of glutaminic and aspartic acids than obtained by the older methods, and it is believed that the results are quantitative.

The method applies equally to proteins which contain small percentages of either of the two amino-acids, and only a small quantity of protein is needed.

Addendum.

The foregoing description applies to the method as it has been used up to the present. Subsequent work, published in another paper [1914], has shown that at such temperatures as the solutions attained when heated on the water bath from time to time during the operations, glutaminic acid is transformed to some extent into pyrrolidonecarboxylic acid. Evidence has already been given to show that the pyrrolidonecarboxylic acid found when applying the method to caseinogen was formed in this way. A further trial is therefore in progress wherein the temperature at any stage of the

operations will not be allowed to exceed 45°. There can be very little doubt that these extra precautions will result in the isolation of the whole of the glutaminic acid as such.

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LIII. THE TRANSFORMATION OF GLUTAMINIC ACID INTO *l*-PYRROLIDONECARBOXYLIC ACID IN AQUEOUS SOLUTION.

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In another paper [1914] an account will be found of a new method for the estimation of aspartic and glutaminic acids in the hydrolytic products of proteins, depending upon the insolubility of their calcium salts in alcohol. In studying the influence of lime upon glutaminic acid in aqueous solution at higher temperatures it was found that the amino-nitrogen content had been considerably reduced. The experiments described below were carried out in order to investigate this matter. The study of the behaviour of the new product when treated in aqueous solution with lime and alcohol was also an object of the experiments.

The glutaminic acid hydrochloride used in this and the other experiments described in this paper was a recrystallised specimen obtained from a caseinogen hydrolysis. Its purity is shown by the following analysis:

	Found per cent.	Calculated per cent.
Carbon	32·61	32·69
Hydrogen	5·80	5·45
Amino-nitrogen ..	7·74	7·63

25 g. glutaminic acid hydrochloride were dissolved in 700 cc. water, 28 g. of pure calcium oxide (about three times the equivalent) were added, and the mixture left in the autoclave for 2·5—3 hours at 140—145°.

The excess of calcium oxide was filtered off, and the liquid was reduced to about 100 cc. at 45° and 15 mm. Rectified spirit (97 % alcohol) was then

added in small quantities at a time with shaking, until no further precipitate resulted. The precipitate was filtered off and washed with rectified spirit, and by extraction with cold water was separated into two fractions, one of which was difficultly soluble and the other easily soluble. These two fractions and the alcoholic filtrate were treated separately as follows:

Fraction *A*, the difficultly soluble portion;
„ *B*, the easily soluble portion;
„ *C*, the alcoholic filtrate.

The calcium was quantitatively removed from fractions *B* and *C* by means of oxalic acid, the alcohol having previously been evaporated from fraction *C*. The calcium was precipitated from fraction *A* as the sulphate by digesting with a slight excess of normal sulphuric acid.

Treatment of Fractions A *and* B. The solutions obtained from these two fractions were then treated separately as follows:

Very small amounts of hydrochloric acid were removed by means of silver sulphate solution. The excess of silver sulphate was decomposed with sulphuretted hydrogen, and the filtrates were freed from sulphuretted hydrogen by evaporation to half bulk on the water bath. The sulphuric acid was then quantitatively removed by means of baryta.

Separation of a substance soluble in cold glacial acetic acid. The filtrates from the barium sulphate were then evaporated to dryness, and dried in the water oven. The residues were extracted with cold glacial acetic acid by triturating in the evaporating dishes with a pestle. The insoluble portions were filtered off by suction and washed on the filter with cold glacial acetic acid. The acetic acid was evaporated slowly from the filtrates leaving gummy residues which gradually crystallised on standing a long time over potash. These, as well as the insoluble portions, were dried and weighed.

The following weights of dried substances were obtained:

	Insoluble in cold glacial acetic acid	Soluble in cold glacial acetic acid
Fraction *A*	6·76 g.	0·86 g.
„ *B*	1·8 g.	4·1 g.

The 6·76 g. portion of Fraction *A* when crystallised from water gave characteristic crystals of glutaminic acid, which melted at 213–214°. The 1·8 g. portion of Fraction *B* also gave the correct melting-point for glutaminic acid.

Treatment of the 4·1 g. portion of Fraction B.

The amino-nitrogen content of this acetic acid soluble portion was estimated by the nitrous acid method.

0·1405 g. gave 2·15 cc. nitrogen at 17° and 747 mm. equal to 0·87 per cent. amino-nitrogen.

This portion was again extracted with a small quantity of glacial acetic acid, filtered by suction, and washed on the filter with glacial acetic acid, the washings, however, were kept separate. The insoluble part when dried weighed 0·61 g.

The acetic acid was slowly evaporated from this main filtrate by allowing the dish to remain on a warm water bath. When reduced to a small bulk the very slightly coloured gummy liquid was placed in a desiccator over strong potash solution. Large crystals separated on standing a long time, the whole of the gummy liquid eventually crystallising completely, the crystals all having the same appearance. These crystals were stirred with about 1 cc. cold glacial acetic acid, filtered by suction, and washed with a mere trace of acetic acid. The larger crystals were left on the filter in a colourless state. The object of this separation was to obtain a specimen completely devoid of light brown colour so that its specific rotatory power could be determined with accuracy. The weight of colourless crystals obtained was 0·47 g. A portion taken from this specimen commenced to soften at 142° and completely melted without colouration at 158°.

The filtrate from the colourless specimen again crystallised completely when treated in the manner already described, and portions were taken from this for analysis. The weights given below were constant at 100°.

The following results were obtained:

0·1449 g.; 0·2520 g. CO_2; 0·0748 g. H_2O; C = 47·43; H = 5·73 per cent.

0·1385 g. gave, by Dumas' method, 13·5 cc. nitrogen at 17° C. and 740 mm.; N = 11·00 per cent.

0·3023 dissolved in 24·76 g. glacial acetic acid gave 0·34° depression of the freezing-point by the cryoscopic method, giving a molecular weight of 140.

The colourless specimen was used for the following determinations:

0·3965 g. was dissolved in water and made up to 20 cc. at 20°. The amount of rotation obtained in a 200 mm. tube was − 0·45°: $[\alpha]_D^{20°} = -11·35°$.

The amino-nitrogen was determined in 7·05 cc. of the solution used for the rotation, equal to 0·1398 g. substance: 0·2 cc. nitrogen was obtained by the nitrous acid method.

5 cc. of the same solution, equal to 0·0991 g. substance required 1·5 cc. N/2 soda for neutralisation to delicate litmus paper. The calculated equivalent of N/2 soda is 1·53 cc. The carboxylic group was therefore exercising its full acidity.

On comparing the figures given in the following table, it will be seen that the substance was pyrrolidonecarboxylic acid. The calculated figures for pure glutaminic acid from which the substance was made are also shown side by side.

	Calculated for glutaminic acid per cent.	Found per cent.	Calculated for pyrrolidonecarboxylic acid per cent.
Carbon	40·83	47·43	46·51
Hydrogen	6·12	5·73	5·43
Nitrogen	9·52	11·00	10·85
Amino-nitrogen ..	9·52	traces	*nil*

The specific rotatory power −11·35° agrees very closely with that found by Abderhalden and Kautzsch [1910], who give figures for *l*-pyrrolidonecarboxylic acid ranging from −10·8° to − 11·52°.

The substance was soluble in alcohol, strongly acid to litmus, and its melting-point agrees with that found by Menozzi and Appiani [1892] and also by Abderhalden and Kautzsch [1910] for *l*-pyrrolidonecarboxylic acid. The carbon is 0·9 per cent. too high, but all the other facts appeared to be so conclusive that a repetition of the combustion was thought to be unnecessary.

Treatment of Fraction C.

After filtering off the calcium oxalate, the hydrochloric acid was removed by means of silver sulphate solution. Excess of silver and sulphuric acid were then removed in the manner previously described. The liquid was evaporated to dryness, and the residue was dried and found to weigh 3 g. This weight would have been undoubtedly less if more water had been removed previously to precipitating the calcium salts with alcohol. This 3 g. was not extracted with cold glacial acetic, as a determination of its amino-nitrogen content appeared to give all the information required.

0·1315 g. gave by the nitrous acid method 4·8 cc. nitrogen at 15·5° and 761 mm. equal to 2·13 per cent. amino-nitrogen.

Assuming this substance to consist of a mixture of glutaminic acid and pyrrolidonecarboxylic acid, and calculating the amino-nitrogen as present

in glutaminic acid, the 3 g. contains 0·67 g. glutaminic acid and 2·33 g. pyrrolidonecarboxylic acid.

The weights of the two substances separated at the different stages are given in the following table:

	Insoluble in cold, glacial acetic acid	Soluble in cold, glacial acetic acid
Fraction *A* ..	6·76 g.	0·86 g.
„ *B* ..	2·41 „	3·49 „
„ *C* ..	0·67 „	2·33 „
Total	9·84 „	6·68 „

The 6·68 g. pyrrolidonecarboxylic acid corresponds to 7·61 g. glutaminic acid. 43—44 per cent. of the glutaminic acid originally taken had therefore been converted into pyrrolidonecarboxylic acid.

Transformation of glutaminic acid into l-*pyrrolidonecarboxylic acid in aqueous solution in the presence of excess of calcium hydroxide, at* 100°.

1 g. portions of glutaminic acid hydrochloride were mixed with 1·2 g. pure calcium oxide and 28 cc. water, *i.e.* in the same proportions as in the previous experiment. These mixtures were boiled in small flasks fitted with reflux condensers, on a sand bath, for different lengths of time. When boiling, the temperature recorded on a thermometer with its bulb immersed in the liquid was 100°. The liquids were cooled, and sufficient acetic acid added to dissolve the excess of calcium oxide. The solutions were then made up to 50 cc., and the amino-nitrogen determined in 10 cc. portions by the nitrous acid method.

A further similar mixture of the substances in the same proportion was kept at 40—45° for 8 hours, and the amino-nitrogen determined in the same way.

The results obtained are shown in the following table:

Weight of substance taken	Temperature	Time	Per cent. amino-nitrogen	Percentage transformed
0·9925 g.	100°	2·5 hrs.	6·45	16·66
0·9955 „	„	5 hrs.	6·17	20·29
0·9933 „	„	7·5—8 hrs.	5·91	23·64
0·9820 „	40—45°	8 hrs.	7·70	*nil*

The amino-nitrogen content of the glutaminic acid hydrochloride originally taken was 7·74 per cent. and the percentages shown had therefore been transformed.

The experiment was repeated using 0·16 g. of a specimen of free glutaminic acid (M.P. 213–214°), the other ingredients being in the same proportion,

viz., 0·24 g. calcium oxide and 5·6 cc. water. After 5 hours boiling, the amino-nitrogen present in the solution was determined. There resulted 20·7 cc. at 19° and 763 mm. equal to 7·47 per cent. amino-nitrogen. Pure glutaminic acid would have contained 9·52 per cent.; 21·53 per cent. of the glutaminic acid had therefore been transformed.

In order to show that the same substance, viz., pyrrolidonecarboxylic acid, had been formed, the solutions left over from the amino-nitrogen determinations were combined. The calcium was removed by means of oxalic acid, and the hydrochloric acid by silver sulphate solution. The excess of silver and sulphuric acid were removed by means of sulphuretted hydrogen and baryta. The liquid was evaporated to dryness, the residue dried, and extracted with cold glacial acetic acid. The small amount of gummy liquid obtained by removing the acetic acid from the extract, crystallised very slowly when allowed to stand over potash. The crystals obtained were exactly similar in appearance to those isolated in the previous experiment. The whole of the preparation was thoroughly dried in a vacuum desiccator over calcium chloride, and the amino-nitrogen determined in a portion of the dry substance by the nitrous acid method. The following result was obtained:

0·1865 g. gave 3·45 cc. nitrogen at 16° and 766 mm., equal to 1·09 per cent. amino-nitrogen.

Effect of other bases.

It was thought, at this stage, that baryta would have the same effect as lime. On carrying out a similar experiment substituting for the lime its equivalent of baryta, however, all the glutaminic acid remained unchanged.

Weight of glutaminic acid hydrochloride taken	Temperature	Time	Per cent. amino-nitrogen
0·9940 g.	100°	5 hrs.	7·63

The effect of substituting other bases for the lime was then ascertained. A pure specimen of the free glutaminic acid was used for this purpose, the amino-acid, water and base being in exactly the same proportion, *i.e.* 0·34 g. to 4 equivalents of the base made up to 12 cc. with water. These mixtures were boiled on a sandbath for 5 hours, under a reflux condenser and made up to 20 cc. with water. The amino-nitrogen was then determined in an aliquot portion. The following results were obtained:

Base	cc. taken from the 20 cc.	Nitrogen obtained, cc.	Temperature and pressure	Amino-nitrogen per cent.	Per cent. changed
$Sr(OH)_2$ ..	9·9	27·85	20° 760 mm.	9·47	*nil*
NaOH ..	9·75	27·7	21° 760 ,,	9·51	,,
NH_4OH ..	10·0	37·1	20·5° 760 ,,	10·4	,,

According to van Slyke [1912] 38·9 per cent. of the nitrogen of the ammonia is liberated by nitrous acid in 5·5 minutes. Applying this correction for the ammonia found present in the solution after the five hours boiling the figure 10·4 was calculated. Although this result is only approximate it suffices to show that no change occurs in the presence of ammonia.

The outstanding difference between calcium hydroxide and the hydroxides of the other alkaline earths is one of solubility in water.

Thus calcium hydroxide is soluble only to the extent of 0·08 per cent. at 100°, while the other bases are very soluble at 100°. Of the three extra equivalents of lime in the 12 cc. solution only about 9 milligrams would be soluble. In the case of the baryta and strontia, however, all the excess would be dissolved. Lactam formation from a salt of glutaminic acid not hydrolytically dissociated appeared to be unlikely. It was therefore concluded that the excess of baryta, strontia and soda were preventing hydrolytic dissociation of the salts. In the case of the lime there was insufficient in solution to do this. The calcium salt of pyrrolidonecarboxylic acid also present would be dissociated, and an equilibrium obtained after 21 per cent. of the glutaminic acid had been transformed.

For this view to be tenable some of the glutaminic acid should be changed, if only one equivalent of baryta were used instead of four. The following experiment was therefore carried out to test this point.

A mixture of 0·17 g. glutaminic acid, 0·375 g. barium hydroxide (one equivalent) and 6 cc. water was boiled for 5 hours under a reflux condenser, and the whole of the solution used for the amino-nitrogen determination with the following results:

Nitrogen	Per cent. Amino-N.	Per cent. changed
24·3 cc. at 20·2° and 768·5 mm.	8·26	13·24

Transformation in aqueous solution.

Having thus established that hydrolysis of the salts of glutaminic acid, involving the presence of the free acid in the equilibrium mixture, appeared to be a condition essential to the formation of the lactam from those salts, there was no apparent reason why the change should not occur simply in aqueous solution, with no base present. This contention was supported by evidence obtained previously when making a preparation of the free acid from its hydrochloride. On this occasion 10 g. of the hydrochloride equal to 8 g. of the free acid were dissolved in water and to this solution heated in a flask on the water bath hot silver sulphate solution was added

until in slight excess. The excess was removed by sulphuretted hydrogen and the filtrate evaporated to half its bulk, on the water bath. Sufficient baryta was then added to the hot liquid to combine quantitatively with the sulphuric acid. After allowing to stand on the water bath until the barium sulphate could be easily filtered, the barium sulphate was removed, and the liquid evaporated on the water bath to crystallisation. The following fractions were obtained:

Crop 1	white	4·29 g.	M.P. 213—214°
„ 2	„	1·49 „	„ 198—199°
Mother liquor evap. to dryness and dried	light brown colour	1·55 „	Softening commenced at about 130°, all melted by 151°

The 10 g. glutaminic acid hydrochloride was taken from a very good specimen, the analysis of which is given on page 481.

The carbon, hydrogen and amino-nitrogen were then determined in the mother liquor portion of 1·55 g., with the following results:

0·1353 g.; 0·2225 g. CO_2; 0·0707 g. H_2O.

0·1580 g.; 7·4 cc. nitrogen at 19° and 764 mm. by the nitrous acid method.

	Calculated for glutaminic acid	Found	Calculated for pyrrolidone-carboxylic acid
Carbon	40·83	44·85	46·51
Hydrogen	6·12	5·81	5·43
Amino-nitrogen ..	9·52	2·71	*nil*

The 1·55 g. evidently consisted of a mixture of glutaminic acid and pyrrolidonecarboxylic acid, as a calculation based on the carbon content showed 29·2 per cent. of glutaminic acid present in the mixture, and on the amino-nitrogen content, 28·5 per cent.

It has already been shown that the carboxylic group of pyrrolidone-carboxylic acid exercises its full acidity when titrated with N/2 soda using delicate litmus paper. Osborne has shown that one of the carboxylic groups of glutaminic acid is neutralised by soda to delicate litmus paper. The amount of soda required to neutralise equal weights of these two acids should therefore be about the same.

The titration of a portion of the 1·55 g. gave a result which agreed with these facts.

As the glutaminic acid hydrochloride originally taken for this experiment gave very satisfactory figures on analysis, the conclusion arrived at was that this 1·1 g. pyrrolidonecarboxylic acid had been produced from glutaminic acid during the repeated heatings on the water bath when in aqueous solution.

As all the facts pointed to the change occurring in aqueous solution the following experiments were carried out.

Two portions of 0·34 g. taken from different specimens of glutaminic acid both melting at the correct temperature were each dissolved in 12 cc. water and boiled on a sand bath for 5 hours under a reflux condenser. The solutions were made up to 20 cc. and the amino-nitrogen determined in 10 cc. by the nitrous acid method.

The following results were obtained :

	Nitrogen cc.	Temperature and pressure	Amino-nitrogen per cent.	Per cent. changed
Specimen *A* ..	13·0	20° & 763 mm.	4·39	53·89
„ *B* ..	13·3	22·2° & 762 „	4·44	53·37

The amount of rotation was determined in a 200 mm. tube in the solution from specimen *B* before using it for the amino-nitrogen determination.

A reading of $-0·01°$ was obtained, the liquid being practically inactive, $[\alpha]_D^{20°} = -0·3°$.

The specific rotatory power of the original glutaminic acid Specimen *B* was also determined and found to be $[\alpha]_D^{20°} = +11·03°$.

The specific rotatory power of *l*-pyrrolidonecarboxylic acid previously found was $-11·35°$. Abderhalden [1909] gives $+10·5°$ for glutaminic acid in aqueous solution.

It is therefore evident that somewhere about half the glutaminic acid is converted into *l*-pyrrolidonecarboxylic acid by boiling the aqueous solution for 5 hours.

The next experiment was designed for the purpose of finding the effect of evaporating solutions containing glutaminic acid on the water bath. With this object in view 0·34 g. was dissolved in 150 cc. water and evaporated to dryness on the water bath. The time taken was 2·5 hours and the temperature of the liquid ranged from 81 to 83°.

When dry, the substance was dissolved in a little normal soda solution and made up to 20 cc. The amino-nitrogen was then determined in 10 cc. with the following results :

cc. Nitrogen	Temperature and pressure	Amino-nitrogen per cent.	Per cent. changed
27·2	18·6° & 761 mm.	9·23	3·1

The influence of temperature is therefore considerable. This gives an explanation for the fact that only 1·1 g. pyrrolidonecarboxylic acid appeared as the result of the repeated evaporations on the water bath, carried out in

the course of working up the 10 g. glutaminic acid hydrochloride already described.

The effect of acids upon the transformation.

Experiments were then carried out to show the effect of acids upon the transformation. 0·17 g. portions and the required amounts of the various acids were in each case made up to 6 cc. with water and the solutions boiled on the sand bath for 5 hours under reflux condensers. The amino-nitrogen was then determined in the whole of each solution. The results are given in the following table:

Acid	Ratio of the acid to glutaminic acid in equivalents	Percentage of acid in the solution	Amino-nitrogen obtained cc.	Temperature and pressure	Amino-nitrogen; percentage of original substance	Per cent. changed
Acetic ..	4	4·6	10·5	19° & 760 mm.	3·55	62·7
Hydrochloric	2	1·38	22·3	22 & 768	7·51	21·12
Sulphuric ..	4	3·72	21·85	19·8 & 768	7·44	21·85
„ ..	2	1·86	16·9	18·5 & 771·5	5·82	38·87
„ ..	8	7·44	25·85	20·5 & 768·5	8·78	7·77

In calculating the number of equivalents in Column 2 of this table, glutaminic acid is considered as a mono-acid base, having regard to the function of its amino-group.

It will be noted that the inhibiting effect upon the lactam formation appears to correspond with the strength of the acid employed. Thus two equivalents of sulphuric acid have the same effect as one equivalent of hydrochloric acid. In the case of the acetic acid the liquid was boiled for 6 hours instead of 5 by mistake. At any rate, however, it may be said that weak acids such as acetic acid have no inhibiting effect. As glutaminic and aspartic acids occur together in proteins, the influence of aspartic acid was tested: 0·17 g. glutaminic and 0·17 g. aspartic acid were together dissolved in 12 cc. water and boiled for 5 hours as before. The amino-nitrogen estimation showed that 53·57 per cent. of change had taken place. It therefore appears that aspartic acid exercises no influence, as the same change was produced in water alone. It is, however, noteworthy that twice as much water *i.e.*, 12 cc. instead of 6 cc., was used for the treatment of this 0·17 g. glutaminic acid.

The figures obtained for the sulphuric acid show that as the amount of acid present in the solution increases, the inhibiting effect also increases, and it may be gathered that when the solution contains about 8 per cent. sulphuric acid, no change at all would occur. It appears reasonable to assume that the same result would be obtained with 3 per cent. hydrochloric acid present.

Reconversion of ring-nitrogen to the amino-form by means of strong hydrochloric acid.

In order to ascertain whether the reverse change, *i.e.*, *l*-pyrrolidonecarboxylic acid into glutaminic acid, occurs on using stronger solutions of a mineral acid, the following experiment was carried out:

0·3285 g. of *l*-pyrrolidonecarboxylic acid was boiled for 4 hours with three times the quantity of strong hydrochloric acid. The liquid was then made up to 20 cc. and the amino-nitrogen determined in 10 cc.

Nitrogen obtained, cc.	Temperature and pressure	Amino-nitrogen percentage of original substance	Per cent. changed
28·6	14·3° C. & 760 mm.	10·24	94·4

The original *l*-pyrrolidonecarboxylic acid contained practically no amino-nitrogen (0·14 g. gave 0·2 cc.). It is therefore obvious that strong hydrochloric acid hydrolyses it to glutaminic acid.

A further experiment, using 5 to 6 times the quantity of hydrochloric acid and boiling for 8 hours, gave a change of 92·2 per cent. of the pyrrolidone carboxylic acid into glutaminic acid. The time required for the complete change has not yet been determined.

The solutions became brown in colour as the boiling continued, and it therefore appears possible that traces of the pyrrolidonecarboxylic acid are decomposed. This colouration appears very frequently when dealing with solutions of this substance.

Abderhalden and Kautzsch [1910] have also shown that pyrrolidonecarboxylic acid is converted into glutaminic acid by heating with hydrochloric acid. They succeeded in obtaining glutaminic acid hydrochloride from the resulting solution.

Secondary formation of pyrrolidonecarboxylic acid in solutions of the products of the hydrolysis of proteins.

As *l*-pyrrolidonecarboxylic acid is hydrolysed to glutaminic acid by boiling with three times the quantity of hydrochloric acid for 4 hours, it is very difficult to conceive of its presence in the liquid arising from a 48 hours' hydrolysis of a protein with strong hydrochloric acid. Up to the present, no experiments similar to those described have been carried out with the inactive pyrrolidonecarboxylic acid, but it is a reasonable assumption that this also will be hydrolysed by strong hydrochloric acid. It has been shown in this paper how pyrrolidonecarboxylic acid may be formed

from glutaminic acid in aqueous solutions and even in solutions containing appreciable percentages of the mineral acids, as well as when solutions presumably containing a hydrolysed salt of glutaminic acid are heated. There are therefore several ways open for the secondary formation of pyrrolidonecarboxylic acid during the treatment of the products of protein hydrolysis. From these considerations it would therefore appear that *l*-pyrrolidonecarboxylic acid is a secondary substance when obtained from the products of the hydrolysis of proteins by hydrochloric acid.

The fact that an increased yield of glutaminic acid hydrochloride generally results when any solution of amino-acids containing glutaminic acid is boiled with strong hydrochloric acid before saturating with hydrochloric acid gas is very probably also accounted for by a re-transformation of pyrrolidonecarboxylic into glutaminic acid.

That even prolonged boiling with hydrochloric acid leaves glutaminic acid quite untouched, is shown by the following experiment:

0·2972 g. pure glutaminic acid was boiled for 48 hours with three times the quantity of hydrochloric acid in a small bulb to which a long tube condenser was sealed. The solution was made up to 20 cc. and the amino nitrogen determined in 9·65 cc. equal to 0·1434 g. glutaminic acid:

Nitrogen obtained, cc.	Temperature and pressure	Amino-nitrogen per cent.	Per cent. changed
23·6	19·5° C. & 761 mm.	9·49	*nil*

Summary.

1. Glutaminic acid is converted into *l*-pyrrolidonecarboxylic acid, to a large extent when its aqueous solution is boiled for a length of time.

2. The change also occurs, but to a much smaller extent, at 81–83°, the temperature of a liquid evaporated in an open dish on the water bath.

3. The same change occurs on boiling an aqueous solution of a salt of glutaminic acid, but to a smaller extent than in an aqueous solution of the amino-acid.

No change takes place when excess of the base is present, probably because hydrolysis of the salt is prevented.

4. Very weak acids have no inhibiting effect upon the lactam formation. The mineral acids, however, do inhibit the change to an extent proportional to their strength and to the amount present. About 8 per cent. sulphuric

acid and about 3 per cent. hydrochloric acid present in the solution of glutaminic acid inhibit the change altogether.

5. The reverse change, viz., of *l*-pyrrolidonecarboxylic to glutaminic acid occurs on boiling with strong hydrochloric acid.

6. Glutaminic acid is unaffected by boiling with strong hydrochloric acid.

7. Pyrrolidonecarboxylic acid may be formed from glutaminic acid in many of the operations involving evaporation or heating when in aqueous solution, during the working up and separation of the products of protein hydrolysis. When found amongst these products, there appears to be no doubt as to its secondary nature.

8. *l*-Pyrrolidonecarboxylic acid is precipitated as the calcium salt by adding excess of alcohol to its aqueous solution previously made alkaline with calcium oxide; when present with glutaminic acid the two calcium compounds are precipitated together.

9. These two substances are also precipitated by adding basic lead acetate solution, and then excess of alcohol, to their aqueous solutions.

10. Glutaminic and *l*-pyrrolidonecarboxylic acids have been obtained in a state of purity by means of two successive extractions of their mixture with cold glacial acetic acid.

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LIV. THE ACTIVATION OF TRYPSINOGEN.

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In a previous paper I showed [1901, 2] that if minced pancreas were kept at room temperature with water or saline solution in presence of chloroform, or with dilute alcohol, the extracting liquid at first contained little or no free trypsin and rennin, but that after a variable time, it might be 5, 12, or 19 days, it suddenly began to develop activity, and in the course of the next few days reached its maximum tryptic and rennetic power. This clearly pointed to an auto-catalytic reaction, and I supposed trypsin itself to be the activator. Small quantities of it, liberated spontaneously from the trypsinogen, would in such a case activate the extract with rapidly accelerating velocity. In seeming proof of such a hypothesis I found that small quantities of active extracts containing trypsin had a very marked activating power upon inactive extracts, whilst a repetition of these experiments with pancreatic juice gave a similar result [1902, 1913]. Mellanby and Woolley [1912] showed that extracts of intestinal mucous membrane containing enterokinase, when added to pancreatic juice, liberated the trypsin at a rate which was slow at first, but which increased with rapidly accelerating velocity to a maximum as the end of the reaction was approached. This result appeared to me to confirm my own experiments, and to indicate that the later and rapid portion of the activation process is due chiefly to the catalytic action of the trypsin liberated in the earlier stages by the enterokinase. For reasons to be adduced later on, I find this hypothesis to be erroneous, and my fresh experimental data point to the following explanation of this vexed problem: *The trypsin liberated by the enterokinase gradually sets free an enzyme, termed deuterase* (to indicate that it acts secondarily to enterokinase) *from a precursor which is present in pancreatic juice and extracts, and this deuterase is mainly responsible for the later stages of the activation process.* Hence in all my previous

experiments in which I have attributed activating powers to free *trypsin*, I now conclude that they are really due to *deuterase liberated by the trypsin from a pro-deuterase.*

Method of Experiment.

The method of estimation of trypsin adopted has already been explained in former papers [1901, 1; 1913]. It depends on Robert's metacasein reaction, suitably corrected, and as a rule takes 3 to 5 minutes to perform, or exceptionally from 2 to 60 minutes. As previously pointed out, this reaction is a test for the rennin rather than the trypsin, but under most conditions the two enzymes run parallel in activity, and so a measure of one is likewise a measure of the other. My earlier experiments were made with a fibrin digestion method which took 20 or 30 minutes for completion, and though it gave useful results it is clearly unsuitable for estimating rapid activation processes.

In all the experiments described below, unless special mention is made to the contrary, both the extracts and pancreatic juice were brought to a fivefold dilution before activation. To the extracts Na_2CO_3 was added up to 0·05% (*i.e.* a centinormal solution), whilst each sample of pancreatic juice used was titrated against N/10 HCl, using methyl orange as indicator, and its alkalinity after dilution reduced to one of 0·05 % Na_2CO_3 by the addition of suitable amounts of N/10 HCl.

The enterokinase was prepared by scraping off the mucous membrane from the upper half of the small intestine of the cat or monkey, grinding it with a little sand, and mixing thoroughly with two to six times its weight of chloroform water. The extract was filtered off 20 to 40 hours later, and kept with chloroform. All the extracts and pancreatic juice were kept in a refrigerator at a temperature of 1° to 5° when not in use.

The Activation of Pancreatic Juice by Enterokinase.

In the few experiments recorded previously on the time relations of the activation of pancreatic juice by enterokinase, I pointed out that the latter part of the activation showed approximately the same velocity whatever the quantity of enterokinase employed. This velocity is so great that its determination is necessarily somewhat inexact, but the plan adopted in the present experiments was to estimate the time taken over the *final half* of the activation process. It was possible to make two or three trypsin estima-

tions during the most rapid stage, as well as other estimations before and after it. These results were plotted out, and the straight line joining the estimations made during the most rapid stage continued till it reached the point of maximum tryptic activity. That is to say, for the purpose of calculating the velocity of the final half of activation it was assumed that there was no slowing down of the activation process as it tended towards completion. Doubtless some slowing does exist, but it is so slight that no good experimental evidence of it could be obtained.

In Fig. 1 is shown the effect of activating pancreatic juice with cat's enterokinase. The 10 % of extract used took 39 minutes to activate at 37°,

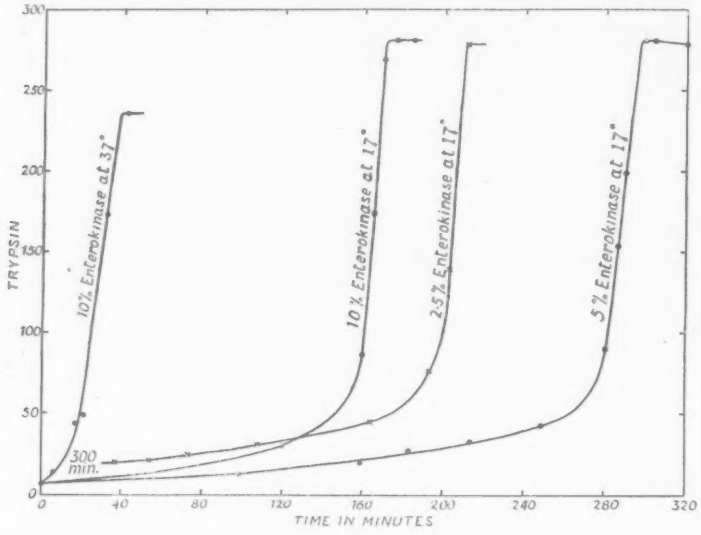

Fig. 1.

but 172 minutes at 17°. These values give a temperature coefficient of 2·10 for each rise of 10° between the limits mentioned, or a similar value to those obtained in most other enzyme actions. Nevertheless temperature does not by any means affect every portion of the curves in the same manner. The last half of the activation process apparently took 12 min. at 37°, but only 8 min. at 17°. The larger value obtained at the higher temperature is doubtless an experimental error due to the difficulty of drawing the slope of the curves correctly when only a small number of experimental results are available, but all the results agree in showing that the latter half of the

activation process is comparatively little affected by temperature. In the other two curves shown in the figure, which indicate the activation produced by 5 % and 2·5 % of enterokinase at 17°, the times taken for the last half of the process were 11 and 8 minutes respectively, or the average time taken at 17° is 9 minutes. In an experiment at 27° the time taken was 13 minutes. Experiments made at 37° with other samples of juice gave the following times for the last half of activation : (*a*) with 10 % of E., 7 min. ; with 5 %, 7 min. ; (*b*) with 6 % of E., 5 min. ; with 3 %, 6 min. ; (*c*) with 10 % of E., 9 min. ; (*d*) with 10 % of E., 5 min. (average of five experiments). Hence the average value obtained at 37° works out at 7 min., whilst the average at 17° and 27° is 10 min. In my previous paper I stated that the time taken for the most rapid doubling of tryptic activity was about 4 min., but if the results there quoted are re-calculated in accordance with the method now used, the time taken over the final half of activation works out at about 5 min.

As will be pointed out again later on, the small influence of temperature upon the final stages of activation of pancreatic juice by enterokinase is due to their being induced for the most part by deuterase, the activating power of which is almost unaffected by temperature.

It will be noted that the time taken for the last half of activation is independent of the quantity of enterokinase used. On the other hand the time taken over the whole process is greatly influenced by it, though not quite in inverse proportion. Thus in the experimental results above recorded activation by 10 % of E. took 172 min., by 5 % took 296 min., and by 2·5 % took 512 min., or in the ratio of 1 : 1·7 : 3·0, instead of 1 : 2 : 4. Another sample of juice was activated at 37° by 10 % of E. in 30 min., and by 5 % of E. in 50 min. Another sample was activated by 10 % of E. in 69 min., and by 4 % of E. in 137 min. On the other hand a fourth sample was activated by 6 % of E. in 32 min., and by 3 % of E. in 83 min., but this exception cannot be held to counterbalance the other three results. Mellanby and Woolley [1913, p. 356] quote one experiment in which the activation time was almost exactly in inverse proportion to the quantity of enterokinase, but in another experiment [1912, p. 375] it was less affected than in any of the results above quoted.

It is of interest to compare the minimum and maximum velocities of activation effected by enterokinase at the beginning and end of its action. For instance, in the above recorded experiment when 2·5 % of E. acted on pancreatic juice at 17°, the tryptic value increased from 7 units to 20 units

in the first 337 minutes, or at the rate of 1 unit in 26 minutes, and as already mentioned it finally doubled itself in 8 min. This increase works out at the rate of 1 unit in 0·057 min., or is 456 times more rapid than the initial rate. Probably it is in reality quite 1000 times more rapid than the real initial rate, as there can be no doubt that the velocity of activation towards the end of the 337 minutes interval was considerably greater than at the beginning. In the experiment with 5 % of E. the velocity observed during the 0 to 99 min. interval was 210 times slower than the final rate observed. With smaller quantities of E. than 2·5 % the difference between the initial and final rates would be greater still, and differences of such an order are seen with most samples of pancreatic juice. Even when kept in a refrigerator they activate themselves spontaneously in about five days. This is presumably due to the presence of minute quantities of enterokinase, which for the first two or three days liberate scarcely appreciable quantities of trypsin, but which finally lead to the familiar rapid activation. Mellanby and Woolley find that extracts of every tissue in the body accelerate the activation of pancreatic juice, and so presumably contain small quantities of enterokinase. The activation of pancreatic extracts observed by me in my original experiments must likewise have been due to the presence of variable traces of enterokinase.

The extreme slowness of the initial rate of activation induced by small quantities of enterokinase, coupled with the fact that the final rate is practically independent of the amount present, clearly points to the existence of a dual reaction in the process. It is not possible to explain such a result by assuming that a co-enzyme is liberated during the course of activation which augments the action of the enterokinase, for in such a case the final velocity would be influenced by the amount of enterokinase present.

The Activation of Pancreatic Extracts by Enterokinase.

For most purposes it is much more convenient to use pancreatic extracts than pancreatic juice, as they can be more readily obtained, and are much more constant in their properties [Vernon, 1901, 2, p. 316]. If fresh sheep's or pig's pancreas be minced up and mixed with four times its weight of glycerin, the extract filtered off one to three weeks later contains large quantities of trypsinogen and only traces of trypsin, and does not activate itself for months if kept in a refrigerator. If, on the other hand, the minced gland be mixed with three parts of glycerin and one of water, the extract begins

to activate itself in about three weeks, and attains its maximum tryptic power in about 5 or 6 weeks. This tryptic power it retains with very slight diminution for many months. If the minced gland be extracted with a mixture of equal parts of glycerin and water it affords an active extract in a fortnight or less, for the larger the proportion of water present in the extracting liquid the quicker the traces of enterokinase in the gland substance exert their action.

The pancreas of animals other than the pig and sheep may yield different results on extraction, partly because the amount of enterokinase contained

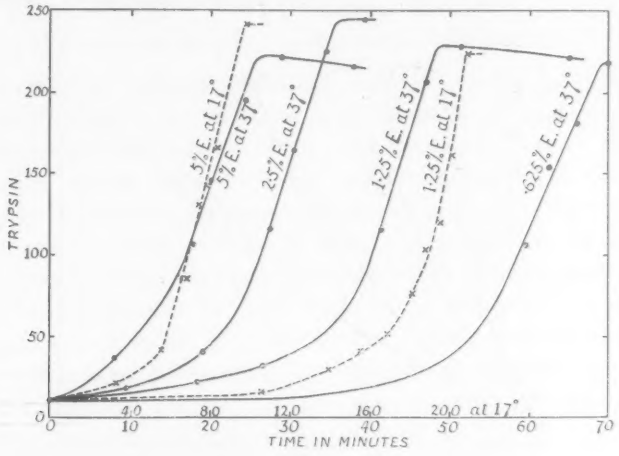

Fig. 2.

in them is variable, and because they may contain an anti-body. For instance, a concentrated glycerin extract of monkey's pancreas was found on two occasions to activate spontaneously in a fortnight. On the other hand a 75 % glycerin extract of ox-pancreas did not activate at all in three weeks.

Inactive glycerin extracts of sheep's pancreas are activated by enterokinase in a very similar manner to pancreatic juice, as can be seen from the curves given in Fig. 2. The times required for complete activation at 37° by 5, 2·5, 1·25 and 0·625 % of E. were 26, 36, 49 and 70 minutes respectively, or were very far from being in inverse proportion to the quantity of enterokinase. The times taken for the last half of activation were 8, 8, 8, and 10 minutes respectively, or were almost independent of the quantity of enterokinase. The results obtained at 17° are represented as dotted line

curves. The time scale on which the values are plotted is four times smaller than that for the values at 37°, and so it can be seen at a glance that the rate of activation at 17° by 5 % and 1·25 % of E. was about four times slower than in the corresponding experiments at 37°. The actual temperature coefficients work out at 1·93 and 2·07 respectively. It will be noted that the slope of the final portion of the dotted line curves is considerably steeper than that of the other curves, and in fact the times taken for the last half of activation by 5 and 1·25 % of E. were 24 and 17 minutes respectively, or two to three times longer than in the corresponding experiments at 37° instead of four times longer. The more marked influence of temperature

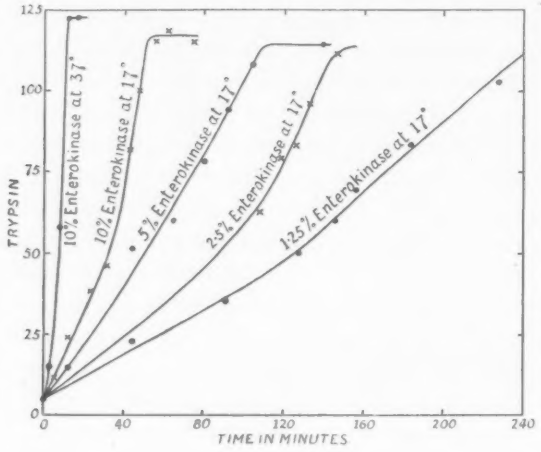

Fig. 3.

on the final stages of activation than in the experiments with pancreatic juice probably indicates that the deuterase factor is relatively less responsible for them.

Extracts of pig's pancreas are not activated by enterokinase in nearly such a typical manner as those of sheep's pancreas. As can be seen from Fig. 3, they much more nearly approach straight lines, or show comparatively little acceleration of velocity in the latter part of the activation. That is to say, the activation is throughout induced very largely by enterokinase, and deuterase is much less important than in the activation of sheep's pancreas extracts. Still it probably accounts for over half of the total activation. This is shown by determining the time taken over the last half of activation, and calculating it as a percentage on the total activation time. The per-

centage times observed are given in the table. They were obtained with various enterokinase preparations, two of which were much more active than the others, and so the percentage amounts of enterokinase given in the first column only hold for about two-thirds of the data. For the remaining third they represent a fivefold diluted solution.

Enterokinase added	Percentage time taken over last half of activation at 37°	17°	1°	Mean
10 %	37, 31	50, 37, 28	22, 20	32
5	—	43, 28	21	31
2·5	42, 32, 28	30, 30	25	31
1·25	34, 22, 22	44	—	30
0·625	21	—	—	21
Mean	30 % at 37°	36 % at 17°	22 % at 1°	

It will be seen that the relative time taken for the last half of activation was about 31 % of the whole time, or was about two-fifths of that required for the first half of activation. Hence even if deuterase were not responsible for any of this first half, it must have induced the larger part of the second half of activation. It will be noted that with a single exception the percentage time is almost constant whatever the amount of enterokinase used for activation. That is to say, with diminishing quantities of enterokinase the slope of the last half of the activation curves changed in almost the same proportion as the time taken for the whole of activation. This points to the final stages of activation being chiefly induced by enterokinase, but evidence indicating that it is largely due to deuterase is afforded by classifying the results according to temperature. In addition to experiments at 37° and 17°, a number were made in which the activating mixture was kept in a vessel surrounded by pounded ice and water, or at a temperature of 0–1°. From the bottom line of the table it will be seen that on an average in these experiments the last half of activation took only 22 % of the total time, as against 33 % taken in the experiments at 17° and 37°. From these data it can be calculated that deuterase must have been responsible for nearly twice as much of the last half of activation at 1° as at 17° and 37°. This result is in accordance with the conclusion that the action of deuterase is almost unaffected by temperature. The considerable difference in the form of the activation curves at 1° and 17° is well shown by comparing Figs. 4 and 3. These results were obtained with extracts of the same pig's pancreas, but the sample used for the experiments shown in Fig. 4 had remained longer in contact with the gland substance before filtration, and so contained much more trypsinogen.

It will be seen from the curves in Figs. 3 and 4 that the time of total activation of pig's pancreas extracts does not vary in inverse proportion to the quantity of enterokinase. Thus the activation times at 17°, with 10, 5, 2·5 and 1·25 % of E. were 52, 109, 148 and 248 min. respectively, whilst at 1°, with 10, 5, and 2·5 % of E. they were 143, 200 and 282 min. respectively. In some observations at 37°, with 10, 2·5 and 1·25 % of E. they were 14, 45 and 65 min. respectively. Experiments with other extracts of pig's pancreas gave similar results. Six sets of experiments made at 37° and 17° with 10 to 1·25 % of E. gave temperature coefficients of 2·07, 1·97, 1·93, 1·93, 1·86 and 1·81, or 1·93 on an average, whilst two sets of experiments at 17° and 1° gave temperature coefficients (likewise for a 10° interval) of 2·24 and 1·94.

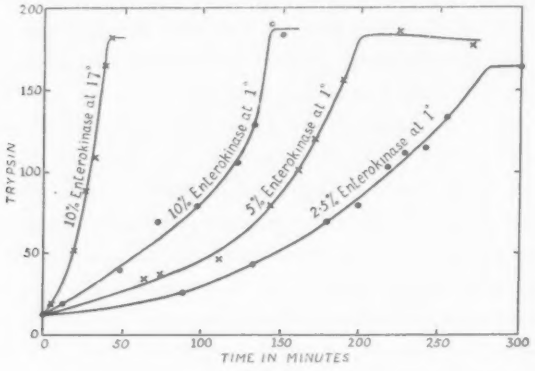

Fig. 4.

Extracts of ox-pancreas gave activation curves similar to those obtained with pig's pancreas. 5 % of E. took 32 minutes to activate at 37° and 108 min. at 17° (temp. coefficient 1·84), whilst the last half of activation took 28 and 31 % respectively on the total activation time.

The difference in the character of the activation curves obtained with extracts of sheep's and of pig's pancreas probably depends on a number of factors. As will be shown later on, various extracts not only contain different proportions of pro-deuterase and of an anti-body, but the rate of liberation of deuterase by trypsin and the action of deuterase on trypsinogen is influenced by various conditions. For instance, products of proteolytic activity retard the formation, or the action, of deuterase much more than the action of enterokinase. In Fig. 5 is shown the effect of adding 0·2 to 3·5 % of Witte

peptone to sheep's pancreas extract which was then activated by 5 % of E. at 37°. It will be seen that with increasing quantities of peptone the rate of the last part of activation, as compared with that of the first part, was more and more diminished. The actual percentage time of the last half of activation on the total time when 0, 0·2, 0·6, 2·0 and 3·5 % of Witte peptone was present was 16, 29, 27, 31 and 46 % respectively. Probably the peptone acts chiefly by retarding the action of the trypsin on pro-deuterase, for it is well known to retard its action on proteins. For instance, I found [1904, 2, p. 350] that 2 % of Witte peptone reduced the digestive action of trypsin on fibrin by 12 %.

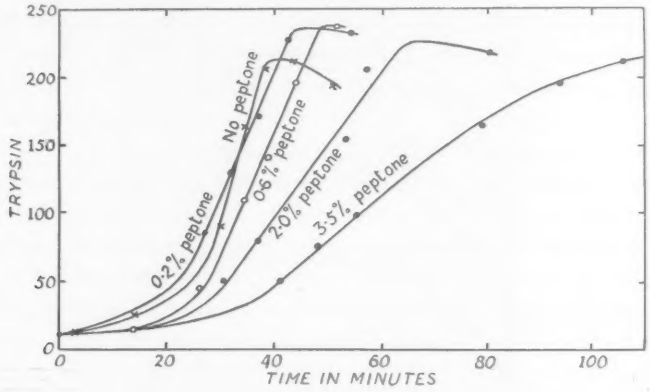

Fig. 5.

Under other conditions the action of enterokinase is much more retarded than that of deuterase. For instance, 5 % of it took 157 minutes to activate a glycerin extract of sheep's pancreas which was diluted with half its volume of water but without the addition of Na_2CO_3, as against 44 minutes when it was diluted to 5 volumes of 0·05 % Na_2CO_3. The last half of activation, however, took only 35 min. as against 15 min., or 22 % of the total activation time as against 34 %. Again, a pig's pancreas extract diluted 50 % with water only, took 203 min. as against the normal 56 min., but took only 12 % of the total time over the last half of activation instead of 28 %. The same extract, diluted 50 % with Na_2CO_3 up to a concentration of 0·08 %, took 139 min. for activation, but only 12 min., or 9 % on the total activation time, over the last half of activation. Presumably this great retardation of the enterokinase is due chiefly to the glycerin itself. Arguing on the

supposition that the gland substance which was extracted by four times its weight of glycerin contained 75 % of water, these activating solutions must have contained about 56 % of glycerin.

The Activation of Trypsinogen by Deuterase.

In previous experiments made with pancreatic juice and extracts I found [1902] that the addition of active pancreatic extracts caused a more rapid activation than the addition of intestinal extracts containing enterokinase. This result I have fully confirmed in my recent experiments.

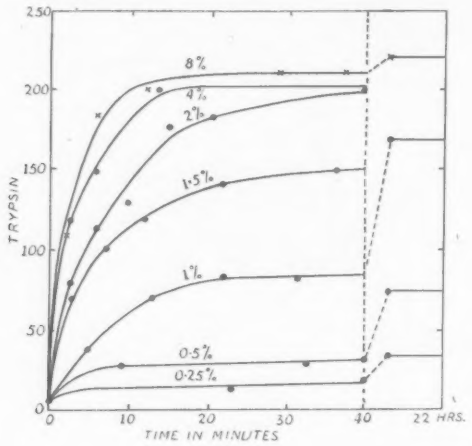

Fig. 6.

In Fig. 6 is shown the effect of adding 0·25 to 8 % of an active glycerin extract of pig's pancreas to an inactive extract at 17°. It will be seen that 8 or 4 % of extract activated almost completely in about 16 minutes, whilst 2 % activated in about 40 minutes. The form of the activation curve is entirely different from that obtained with enterokinase. Activation begins very rapidly and gradually slows down to zero as the available mass of trypsinogen diminishes, or deuterase appears to act logarithmically like most other enzymes. However, its action does not as a rule obey the logarithmic law, for it will be noted that the activation slows down and nearly ceases before the whole of the trypsinogen is converted. This is especially well seen when 1 % or less of deuterase extract was employed. In these experiments the activation almost stopped in the first 10 or 20 minutes, and then continued

with such extreme slowness that even 22 hours later, as can be seen from the data on the extreme right of the figure, a large portion of the trypsinogen was still unconverted. As will be shown later, this very slow and continuous conversion must be due to the action of fresh deuterase gradually liberated by the trypsin from the pro-deuterase contained in the inactive extract.

It should be stated that in these experiments the tryptic value of the active extract added was always deducted from the tryptic value observed. Also it was found that the deuterase continues to activate the trypsinogen of the extract to some extent after the sample has been added to the diluted milk for estimation, though not so rapidly as in the original solution. Hence in plotting out the curves *half* of the time taken for each estimation—which was generally 3 or 4 minutes—was added to the actual time from start of experiment to beginning of the estimation. In the enterokinase experiments no such correction was made, as the enterokinase appeared to cease activating on mixing with the milk.

A large number of activation experiments have been made with different substrates and different deuterase extracts, and they show that no single law of action will suffice to explain them. From evidence which will be adduced later it seems probable that both the trypsinogen and the deuterase in pancreatic extracts exist in various colloidal states, the condition of which can be readily changed, and which greatly influence their interaction. For instance, in Fig. 10 (see below) is recorded another set of experiments made with the same pancreatic extracts as were used in the experiments just described, but three months previously. It will be seen that the activation curves obtained with 0·7 and 1·2 % cf deuterase approach the logarithmic type much more closely than those in Fig. 6. In still another experiment with the same inactive glycerin extract of pig's pancreas, but with a different deuterase extract (obtained by extracting pig's pancreas with two parts of N/10 HCl; slightly over-neutralising 17 hours later with solid Na_2CO_3, and filtering off), [*cf.*, Mellanby and Woolley, 1914, 1], the activation curve was definitely logarithmic in type. It is impossible to determine the total amount of trypsinogen present in an extract with great accuracy, for in the first place the method of trypsin estimation employed is only moderately accurate, and it does not give quite the same results from day to day as the samples of milk vary somewhat in quality. This glycerin extract had on previous occasions developed a maximum tryptic value of 236, 229, 219, 212 and 211, when kept at 17° with deuterase, but in the present experiments developed a value of only 200. Taking this to represent the effective maxi-

mum, and deducting from it the 7 units of free trypsin present in the extract before activation, it will be seen from the table that the results obtained at 17° and 37° roughly follow the logarithmic law for uni-molecular reactions, viz. :

$$K = \frac{1}{t} \log \frac{1}{1-x}.$$

The most exceptional values were those obtained in the two initial estimations. They are probably due to the correction made for the activation which continues during the trypsin estimation being too great.

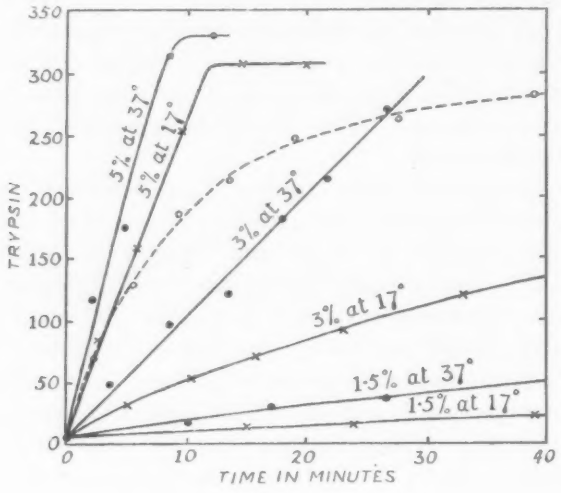

Fig. 7.

At 17° C.			At 37° C.		
Trypsin liberated (x)	Time (t)	K	Trypsin liberated (x)	Time (t)	K
46	3·5 min.	0·034	48	3·25 min.	0·038
69	8·4	0·023	69	8·0	0·024
95	13·0	0·023	101	15·5	0·021
109	18·0	0·020	114	23·75	0·016
127	23·5	0·020	155	35·5	0·020
170	69·0	0·014	182	42·75	0·029

In another series of experiments, made with extracts of sheep's pancreas, the rate of activation as a rule followed the linear law. In Fig. 7 is shown the effect of adding active glycerin extract of sheep's pancreas to inactive extract, and it will be seen that the activation curves are almost straight lines,

and continue so nearly to their completion. Yet these very same extracts, when tested some four months later, gave activation curves more or less midway between the linear and logarithmic types. Also, the inactive extract, at the same time that it was giving linear curves when activated by the active extract of sheep's pancreas, under some circumstances gave nearly logarithmic curves when activated by the HCl extract of pig's pancreas above mentioned. It should be stated that this extract had begun to activate spontaneously immediately it was prepared, but since at the time it was required for this experiment it had not quite completed its activation, a trace of enterokinase (0·1 %) was added to it and it was kept 24 hours before use. The action of 10 % of this mixture is shown in the dotted line curve of Fig. 7. Similar curves of a logarithmic type were obtained on activating with 6 and 4 % of the mixture, but 3, 2, 1·5 and 1 % of it gave absolutely linear activation curves. In their case the available mass of substrate must have been in excess of the maximum upon which the enzyme could act at any moment.

Five days after the HCl extract had activated itself completely its action on inactive sheep's extract was again tested, and 10 % of it, acting at 37° and at 17°, yielded curves almost identical in form and position with those shown on the extreme left of Fig. 7. The changed action seemed to depend partly on the lack of a trace of enterokinase, for after keeping some of the completely activated HCl extract for 24 hours with 0·1 % of E., it again yielded a curve approaching the logarithmic type. On the other hand some of the HCl extract likewise gave logarithmic instead of linear curves when diluted and kept five days with 0·05 % Na_2CO_3. Also, the inactive extract of sheep's pancreas under certain conditions, to be mentioned later, yielded curves very similar to those shown in Fig. 6, whilst it yielded curves about midway between the logarithmic and linear types when activated by active extract of pig's pancreas.

A glycerin extract of ox-pancreas, acted on by an active glycerin extract of monkey's pancreas, gave nearly linear curves (*cf.* Fig. 16).

These very conflicting results suggest that a change in the colloidal condition of enzyme and substrate, and in their manner of interaction, is effected very easily. More striking proof of the existence of different kinds of trypsinogen in pancreatic extracts came to light in quite another way. It was found that the maximum tryptic value induced on addition of enterokinase to inactive extract was always less than when it was kept with active extract (*i.e.* deuterase). The difference in the two maxima

attained varied considerably even in the same extract at different times. For instance, a glycerin extract of pig's pancreas was filtered off from the gland substance after 23 to 26 days' extraction, and was then found to develop a maximum tryptic value of 132 on addition of enterokinase. Four days later the maximum was 160 ; twelve days later it was 187, but three months later it averaged 215 in various experiments. On keeping this same extract with various deuterase extracts it developed an average tryptic value of 221, hence after the three months' interval practically all of its trypsinogen was in the same form. On the other hand a glycerin extract of sheep's pancreas, even after it had been kept three months, developed a maximum tryptic value of only 252, 236, and 244 in various experiments on addition of

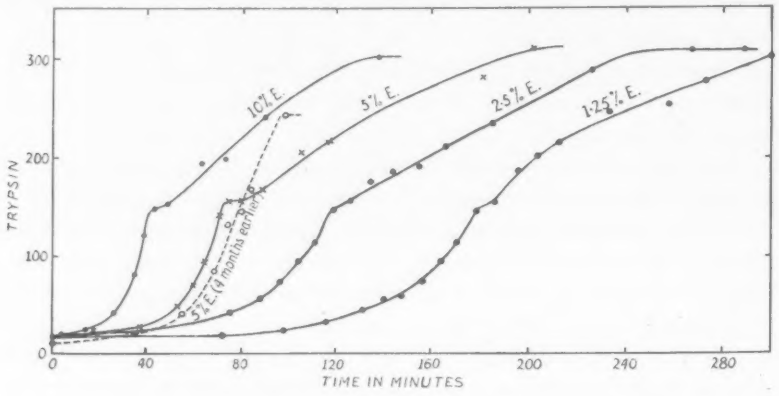

Fig. 8.

enterokinase, but one of 334, 313 and 312 when deuterase was added. That is to say, on an average only 76 % of the trypsinogen was converted into trypsin on the addition of enterokinase. Of course the extract did attain the same maximum after addition of enterokinase as after the addition of deuterase if it were given sufficient time, and if the destruction of the liberated trypsin were avoided by carrying out the experiment at 17° or 1°, but this only occurred slowly and was quite distinct from the rapid activation to a nearly constant level which is so typical of the action of deuterase.

After the glycerin extract just mentioned had been kept another four months in cold storage, it was found that nearly *half* of its trypsinogen had passed into the relatively stable form. This is strikingly proved by the series of experiments recorded in Fig. 8, in which it was acted on by 10 to

1·25 % of enterokinase at 17°. With 10 % of enterokinase the typical activation was completed in 41 minutes, the last half of activation taking only 15 % of the total time. Then the activation of the second half of the trypsinogen proceeded at a rate about seven times slower than the previous maximum rate, and it took about 97 minutes for its completion. The action of 5 % of E. was equally striking, but with 2·25 % and 1·25 % of E. the change in the slope of the curve after activation of the first half of the trypsinogen was not so marked. It will be noted that within the limits of experimental error the slope of the last half of the activation curves is almost the same whatever the amount of enterokinase present, *i.e.*, it is a deuterase effect.

The dotted line curve shows the action of 5 % of E. (another preparation) on this same extract of sheep's pancreas four months previously. It is one of the results already recorded in Fig. 2. Unfortunately the rate of activation in these earlier experiments at 17° was not investigated after the supposed maximum had been reached. It had been investigated in the corresponding experiments at 37°, and was then found to fall off rapidly. The reason is that the trypsin is so unstable at 37° that its destruction rate is greater than its activation rate, when such activation occurs at the slow speed shown by the latter half of the curves in Fig. 8.

The Activation of Pancreatic Juice by Deuterase.

Pancreatic juice is activated by deuterase in the same way as pancreatic extracts. In Fig. 9 (p. 510) is shown the effect of 1 to 3 % of a glycerin extract of pig's pancreas at 37°, and of 3 % at 17°. The curves are rather irregular in form, owing largely to the fact that the final stages are complicated by the activating effect of fresh deuterase liberated from the pro-deuterase present in the juice. The difference in the rate of activation induced by 3 % and by 2 % of deuterase at 37° is so remarkable that it might be thought to be due to some experimental error: but a repetition of the experiment a day later gave almost the same result. The two sets of values obtained with 3 % of deuterase are shown in the figure.

Experiments on the activation of pancreatic juice by the deuterase of activated juice will be described in a subsequent section.

The Temperature Coefficient of Deuterase.

As already mentioned, the activation of trypsinogen by deuterase is only slightly affected by temperature. A striking proof of this statement

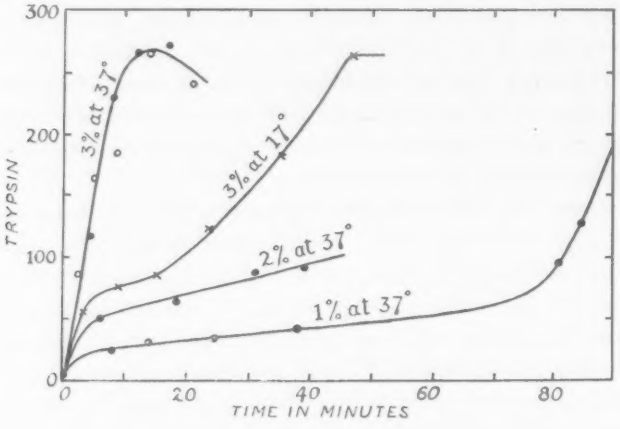

Fig. 9.

is afforded by the table showing the relationship between time and trypsin liberated which was given a few pages back. The average velocity coefficient, K, was 0·0223 at 17°, and 0·0247 at 37°, or nearly the same. A number of

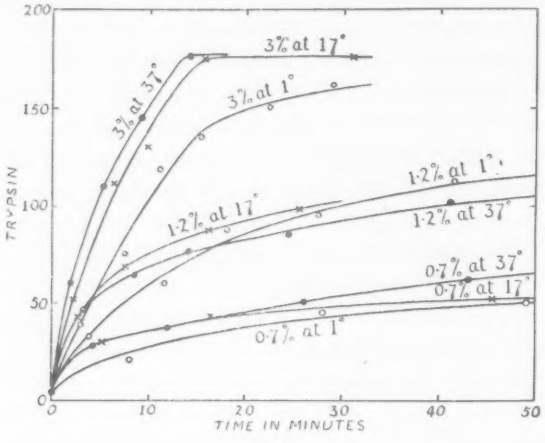

Fig. 10

other experiments in which an active glycerin extract of pig's pancreas was allowed to activate an inactive glycerin extract of pig's pancreas gave almost the same result. One series is shown in Fig. 10, and here we see that though 3 % and 0·7 % of deuterase activated rather more quickly at 37° than at 17°, 1·2 % activated more quickly at 17° than at 37°, or the average of the three sets of experiments indicates that the temperature change was practically without influence. At 1°, however, there is a distinct retardation in the rate of activation, especially in the initial stages. Later on there is an acceleration, and in the experiments with 1·2 and 0·7 % of deuterase we see that 30 minutes after the start the activation was proceeding at about the same rate at 1°, 17°, and 37°. This acceleration is due in part to the greater stability of deuterase at the low temperature, but there must be some other factor concerned, as deuterase is only very slowly destroyed at 17°.

As can be seen from the curves given in Fig. 7, the action of deuterase on extract of sheep's pancreas is considerably affected by temperature; but the actual temperature coefficient is not nearly so high as might be imagined at first sight. It is true that the rate of activation by 3 % of deuterase at 37° was nearly three times greater than at 17°, and by 1·5 % of deuterase, three and a half times greater, but it can be calculated from the curves that 3 % of deuterase at 37° activated the trypsinogen nine times more rapidly than did 1·5 %, and at 17°, fourteen times more rapidly. Hence in order to arrive at the real temperature coefficient, the relative amounts of deuterase required to induce the same rate of activation at 37° and at 17° must be calculated. It is found that 2·5 % of deuterase at 37° would activate at the same rate as 3·5 % at 17°, or that the temperature coefficient for this extract is 1·18.

It is a more accurate method to determine by direct experiment the relative amounts of deuterase required to activate at the same rate at 37° and 17°, and this has been done in two instances. It was found that 2 % of an active glycerin extract of pig's pancreas at 37° activated glycerin extract of ox pancreas at almost exactly the same rate as 3 % at 17°, or that the temperature coefficient was 1·22. Again, 2·5 % of active glycerin extract of sheep's pancreas at 37° activated pancreatic juice at the same rate as 3·0 % at 17°, or gave a temperature coefficient of 1·09.

The temperature coefficients for the activation of trypsinogen by enterokinase and deuterase are collected in the table for the sake of contrast. Only the data obtained between the temperature limits of 17° and 37° are included.

		Temp. coefficient (10°)	
Enterokinase activating	Pancreatic juice	2·10	2·00
	Extract of sheep's pancreas (2·07, 1·93)	2·00	
	„ „ pig's pancreas (2·07, 1·97, 1·93, 1·93, 1·86, 1·81)	1·93	
	„ „ ox pancreas	1·96	
Deuterase activating	Pancreatic juice	1·09	1·12
	Extract of sheep's pancreas	1·18	
	„ „ pig's „	1·00	
	„ „ ox „	1·22	

A large number of experiments were made in which the data were insufficient to give an accurate temperature coefficient, so in order to compare

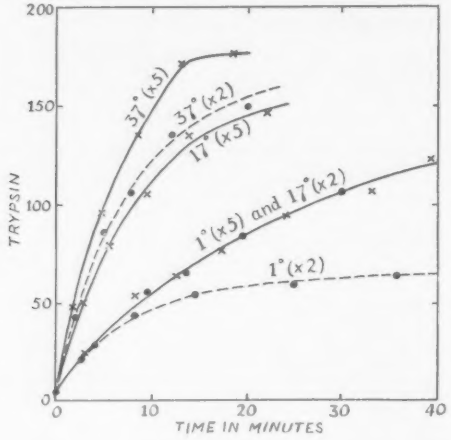

Fig. 11.

the temperature effects the method adopted was to read off from the plotted curves the times taken for liberation of 50 % of the total amount of trypsin set free in 30 or 40 minutes at 37°, and then find the time taken for the liberation of the same amount of trypsin at 17°. The time of liberation of 50 % of trypsin is taken for comparison rather than that of the completion of activation, as it can be estimated more accurately.

The differences of temperature effect observed with extracts of pig's pancreas and of sheep's pancreas appear to depend on the quality of both enzyme and substrate. Taking first the experiments made with glycerin extracts, it was found that when pig's pancreas deuterase activated sheep's pancreas trypsinogen, it took 1·4 times longer at 17° than at 37°, instead of 1·5 to 3·1 times longer as in the corresponding experiments when sheep's

deuterase activated sheep's trypsinogen. In the converse experiment when sheep's deuterase activated pig's trypsinogen it took, in various experiments, 1·2, 1·3 and 2·3 times longer at 17° than at 37°, whereas we have seen that pig's deuterase activated pig's trypsinogen as quickly at the lower temperature as at the higher. Hence the temperature effect depends partly on enzyme, but probably the physical condition of the substrate is the most important factor, and it can readily be modified so as to influence the temperature effect enormously. This is well shown by the two series of curves reproduced in Fig. 11. In one experiment, the results of which are represented by continuous line curves, some of the same glycerin extract of pig's pancreas, which on fivefold dilution with 0·05 % Na_2CO_3 was activated as quickly at 17° as at 37°, was diluted fivefold with water only. It was activated by 5 % of active pig's pancreas extract, and we see that its activation was distinctly slower at 17° than at 37°, and very much slower at 1°. In the other experiment the same extract was diluted with an equal volume of water only, and then it took four times longer to activate at 17° than at 37°, and much longer still at 1°. The curve obtained at 17° happens to be identical in position with the 1° curve of the more diluted extract.

Experiments with the active HCl extract of pig's pancreas previously mentioned gave similar results. When allowed to activate glycerin extract of pig's pancreas it took 1·6 times longer at the lower temperature, and when activating sheep's extract it took, in various experiments made at different times with different amounts of enzyme, 2·2, 2·7, 2·8, 3·8 and 6·4 times longer at the lower temperature.

The Rate of Liberation of Deuterase.

We have seen that the addition of small quantities of active pancreatic extracts to inactive extracts rapidly activates the trypsinogen, but if a sample of this freshly activated extract be added to more inactive extract it is found to possess little or no activating power. For instance, in Fig. 12 we see the effect of adding 3 % of active extract of pig's pancreas to inactive extract at 17°. Activation was almost complete in 30 minutes, and a minute after this time (marked by an arrow in the figure) a sample of the mixture containing 3 % of the extract was added to more inactive extract at 17°, and we see from the lowest curve in the figure that it had extremely little activating power. Another sample of the mixture taken 48 minutes after had distinctly more activating power: one taken 115 minutes after much more activating

power still, whilst a sample taken 175 minutes after had almost as much activating power as the original pancreatic extract. It is concluded, therefore, that a freshly activated extract contains scarcely any deuterase, but that this enzyme is gradually liberated from the pro-deuterase present in the extract by the action of the free trypsin. In proof of this hypothesis, it is found that the rate of development of activating power is dependent on temperature. On repeating the experiment at 1°, it was found that 3 % of the active extract activated the inactive extract to about the same point as that indicated in Fig. 11 in 90 minutes. At times ranging from 145 to 696 minutes later, 3 % samples of it were added to inactive extract at 17°,

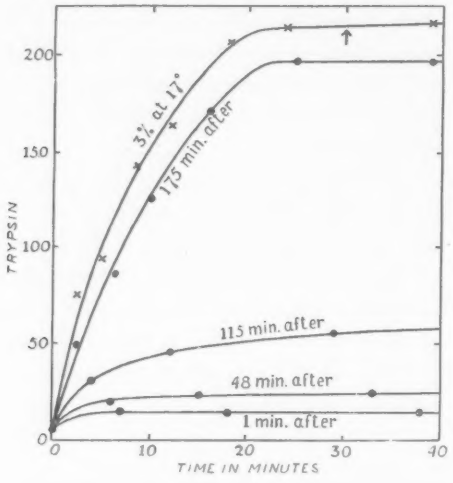

Fig. 12.

and it was found that the activation curves were of the same character as those shown in Fig. 11, except that the rate of generation of activating power in the mixture—which was kept throughout at 1°—was about four times slower than before. This is shown by the curves given in Fig. 13, which indicate the percentage of trypsin liberated in 40 minutes on addition of 3 % samples of the regenerating mixtures to inactive extract. When the results recorded in Fig. 12 are plotted out in this manner, it is seen that the rate of development of activating power gets more and more rapid from start to finish. The results obtained at 1° are plotted out on a time scale reduced fourfold, and we see that the curve obtained is of similar form and position to that obtained at 17° ; *i.e.* that the rate of development of activating

power in freshly activated extracts is four times slower at 1° than at 17°. At 37° it is probably about four times quicker than at 17°, but the results are unsatisfactory owing to the extreme instability of the liberated deuterase at this temperature. Thus an experiment was made in which 3 % of active extract was added to inactive extract at 37°, and it activated it almost completely in 24 minutes. At times ranging from 5 to 139 minutes later 3 % samples of this mixture—kept throughout at 37°—were added to inactive extract at 17°, and the percentages of trypsin liberated in 40 minutes are shown in Fig. 13. We see that though the activating power of the mixture

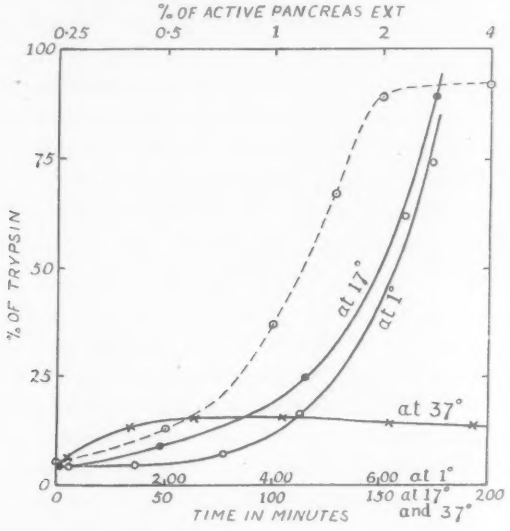

Fig. 13.

at first developed much more rapidly than that kept at 17°, it quickly slowed down, and after 105 minutes began to deteriorate.

The rapidly accelerating rate of formation of deuterase in freshly activated extracts is a mysterious and unexpected phenomenon. It is true that within certain limits the amount of trypsin liberated in an inactive extract on the addition of active extracts increases in greater proportion than corresponds to the extract added. For instance, the curves in Fig. 6 indicate that the addition of 0·25, 0·5, 1 and 2 % of active extract to inactive extract caused the liberation, in 40 minutes, of 12, 28, 79 and 193 units of trypsin respectively, or in the proportion of 1, 2·3, 6·6 and 16·1, instead of 1, 2, 4

and 8: but the rate of acceleration of deuterase formation is much more rapid than this. It occurs in geometrical progression, as is shown by comparing the curves of deuterase formation in Fig. 13 with the dotted line curve. This curve indicates the percentage of trypsin liberated by 0·25 to 4 % of active extract (*cf.* Fig. 6), but plotted out on the logarithmic scale given at the top of the figure. The curve is very similar in form to those indicating the rate of formation of deuterase at 17° and 37°, and hence it follows that this likewise occurs in geometrical progression.

In contradistinction to this geometrical increase in the rate of deuterase liberation is the fact that on addition of small quantities of deuterase to inactive extracts a certain amount of trypsinogen is activated almost at once, but then the activation slows down and continues so slowly as to be incomplete even 24 hours later (*cf.* Fig. 6). One would have thought that the trypsin liberated by the deuterase added would at once begin to liberate more deuterase from the pro-deuterase present, and that in consequence the activation would soon begin to accelerate again as it does in the presence of enterokinase. So far from that, the results recorded in Fig. 6 indicate that 0·5 % of deuterase, though it liberated 26 units of trypsin in 40 minutes, liberated only an extra 42 units in 22 hours. Again, 1 % of deuterase liberated 78 units in 40 minutes and an extra 90 units in 22 hours, though at the end of this time 52 units of trypsin still remained in the zymogen form. Experiments with sheep's pancreas extracts showed that the activation induced by deuterase continued much more steadily than that in pig's pancreas extracts, but even in them no acceleration was observed. The explanation of this result is unknown, but it seems to be dependent on conditions, such as the presence of anti-bodies, which do not obtain in pancreatic juice. As already pointed out, the results recorded in Fig. 9 clearly indicate that the addition of deuterase to pancreatic juice causes not only a rapid initial activation of a portion of the trypsinogen, but after an interval of slow activation this is succeeded by a second acceleration which must be due to the liberation of fresh deuterase. The results obtained in another experiment at 17° are recorded in Fig. 14 (see below), and two of the curves there reproduced show this secondary acceleration. In a similar experiment carried out at 37° the secondary acceleration appeared in every case.

It is evident that if time be given for the deuterase in freshly activated extracts to be liberated, the capacity for activation can be transmitted from extract to extract indefinitely. For instance, 5 % of an active extract of pig's pancreas was used to activate inactive extract of pig's pancreas,

and 4·5 hours later 5 % of this mixture was added to more inactive extract. 15 hours later 5 % of *this* mixture was used to activate still more inactive extract, and 5·5 hours later 5 % of this last mixture was added to still another sample of inactive extract. All the activation curves in the series were practically the same in form.

Experiments with glycerin extracts of sheep's pancreas gave results similar to those obtained with extracts of pig's pancreas. For instance, 5 % of active sheep's extract was added to inactive extract at 17°, and 36 minutes later the addition of 5 % of this mixture to more inactive sheep's

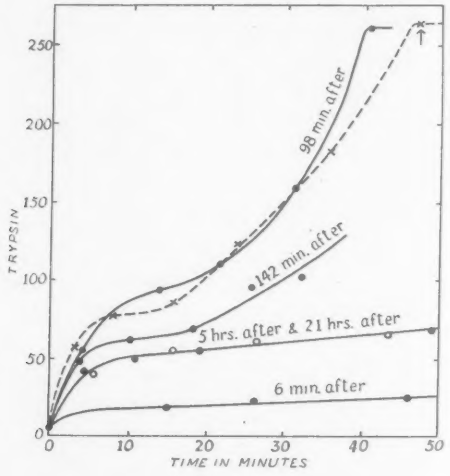

Fig. 14.

extract at 17° liberated 8 units of trypsin; 51 minutes later it liberated 13 units; 150 minutes later 24 units, but 343 minutes later, 165 units. Hence there was a rapid acceleration in the rate of deuterase formation in the latter part of the experiment, as in the previous series of experiments. The activation curves were somewhat similar in form to those shown in Fig. 12, and were not linear like the sheep's pancreas curves shown in Fig. 7.

Pancreatic juice yielded similar results to pancreatic extracts. In an experiment at 17°, 3 % of active extract of pig's pancreas activated the juice completely in 47 minutes, and 6 minutes later 5 % of the active juice was added to inactive juice at 17°, but as can be seen in Fig. 14 its activating power was but small. When it was tested again 98 minutes after it had probably reached its maximum activating power, for when tested 142 minutes

after it was distinctly less active. When tested 5 hours and again 21 hours after it had less activating power still. Hence the curves shown in the figure seem to indicate that the activating power of freshly activated pancreatic juice reaches its maximum two to four times more quickly than in pancreatic extracts, and that the deuterase formed is so unstable as to undergo rapid destruction even at 17°. In a similar experiment at 37° 3 % of active extract activated the juice completely in 13 minutes, and the immediate addition of 5 % of this active juice to inactive juice at 37° had as much activating effect as that shown in the lowest curve in Fig. 14. A sample taken 25 minutes later had nearly as much effect as that shown in the "142 minutes after" curve of Fig. 14, whilst a sample taken 104 minutes after had slightly less effect than that shown in the lowest curve of Fig. 14. Another experiment at 37° showed that the freshly activated juice had more activating power 14 minutes after it had completed activation than either immediately after or 40 minutes after. Hence it is evident that at 37° the deuterase of freshly activated juice is extraordinarily unstable. It is quickly liberated, but within a very few minutes most of it is destroyed.

A Comparison between the Activating and Tryptic Powers of Extracts.

The experimental results thus far described are explicable on the hypothesis which I put forward in previous papers to the effect that trypsin itself is a powerful activator of trypsinogen. I found that fresh pancreatic extracts, rich in trypsin, had relatively much greater activating power than extracts which had deteriorated to some extent in tryptic power as the result of being kept. Also I found that the trypsin (or rennin) in freshly prepared extracts is so unstable that even when kept with water only (and no Na_2CO_3) at 38° about 50 % of it is destroyed in an hour. That in kept extracts is much more stable, and may be destroyed at the rate of only 12 % per hour [Vernon, 1901, 1, p. 196]. Hence I concluded that trypsin exists in various states of stability, and that only unstable trypsin has much activating power. It was suggested to me by Prof. W. M. Bayliss that I had no good evidence that the activating substance was this unstable trypsin, and further experiments supported his contention, for I found that in various freshly prepared active pancreatic extracts there was no relationship between activating power, trypsin content and stability of trypsin. It is quite true that if any extract deteriorates as the result of being kept, its activating power diminishes

much more rapidly than its tryptic power, but this is because the deuterase it contains is much more unstable than the trypsin.

Anything more than a rough comparison between the activating powers of extracts is useless, in that such comparisons do not hold at all closely at different concentrations. A comparison of the activation curves obtained with pig's pancreas extracts and sheep's pancreas extracts which are shown in Figs. 6 and 7 proves this. In experiments made at 37° it was found that 3 % of active sheep's extract activated inactive sheep's extract at the same rate as 1·25 % of active pig's extract, or that its activating power was 42 % as great. A similar experiment at 17° showed it to be 40 % as great. In other experiments made at different times upon inactive pig's extract it was found that 6, 4·5 and 3 % of active sheep's extract activated at the same rate as 2·0, 1·5 and 0·65 % of active pig's extract, or was 33, 33 and 22 % as active in the respective experiments. On an average, therefore, the sheep's extract had only 34 % of the activating power of the pig's extract, in spite of the fact that its tryptic power was almost exactly the same. The HCl extract of pig's pancreas, though it had three-fourths the tryptic power of the glycerin extract, had only a sixth its activating power, whilst a watery extract of pig's pancreas (made by extracting for 42 hours with 2 parts of chloroform water, filtering off and keeping with chloroform) had about half the tryptic power of the glycerin extract, but only a thirtieth the activating power. These experiments with pig's pancreas extracts confirm those formerly made by me [1901, 2, p. 295], in which I found that 0·9 % NaCl extracts of the pancreas of various animals had much less activating power, relative to their trypsin content, than their glycerin extracts.

The most striking evidence of the lack of correspondence between activating power and tryptic power was obtained with the other two extracts recorded

Pancreatic Extract	Relative activating power	Mean	Trypsin	% Trypsin destroyed in 1 hour at 37°
Glycerin extract of pig		100	294	12
,, ,, ,, monkey	83, 62, 52	66	83	16
,, ,, ,, sheep	42, 40, 33, 33, 22	34	293	65
HCl ,, ,, pig		17	218	17
H_2O ,, ,, pig		3·5	144	—
Glycerin ,, ,, ox		1	183	78

in the table. A glycerin extract of monkey's pancreas was found to have only about a fourth as much tryptic power as the glycerin extract of pig's pancreas, but two-thirds its activating power. This relative richness of

the monkey's gland in pro-deuterase may help to explain the fact that both the concentrated glycerin extracts made with the pancreas of healthy Macaque monkeys, which had been killed instantaneously, activated themselves spontaneously within a fortnight of their preparation.

In contradistinction to monkey's pancreas extract, a 75 % glycerin extract of ox-pancreas, though it had nearly two-thirds the tryptic power of the pig's extract, had no activating power at all in most experiments. As there was a slight indication of it in one or two instances, its actual power is recorded in the table as 1 % on that of pig's extract. As will be shown in the next section, the extract did contain some deuterase, but its activating powers were masked by the presence of an anti-body.

The stability of the trypsin in pancreatic extracts was tested by diluting them fivefold with water and Na_2CO_3 to 0·05 %, and keeping for an hour (or two hours) at 37°. Under such conditions no less than 78 % of the trypsin of the ox-pancreas extract was destroyed, and 65 % of that in the sheep's extract. The other extracts were much more stable, and the glycerin extract

Extract	% trypsin destroyed in 1 hr. at 37°	Mean
Glycerin extract of ox pancreas	82, 76, 76	78 %
„ „ „ sheep's „	71, 70, 70, 69, 64, 59, 52	65
HCl „ „ pig's „	21, 19, 17, 12	17
Glycerin „ „ pig's „	27, 26, 23, 23, 22, 21, 21, 14 (two hours)	(22)
„ „ „ monkey's pancreas	17, 16	16

of pig's pancreas had only 22 % of its trypsin destroyed in *two* hours. The stability of the trypsin probably depends on its colloidal state, and so varies according to the manner in which an extract of a gland is prepared, for my earlier experiments with pig's pancreas extracts gave a very different result from the present ones. When tested by a fibrin digestion method it was found that the trypsin of a glycerin extract was destroyed at the average rate of 69 % per hour by 0·4 % Na_2CO_3 at 38°, and at the rate of 39, 32, 24 and 23 % per hour by water only (in this case tested by the metacasein method). That is to say, it was destroyed about three times more rapidly than in the present experiments, if we assume that 0·05 % Na_2CO_3 is rather more injurious than water only. A glycerin extract of sheep's pancreas was destroyed at the average rate of 80 % per hour by 0·4 % Na_2CO_3, and at the rate of 66, 60, 53 and 39 % per hour by water only, or was about as unstable as in the present experiments.

The great instability of deuterase as compared with trypsin is proved by several experiments described in the previous section, in which the activating power of juice kept at 17° and 37° was found to deteriorate rapidly,

though the tryptic power was almost unaffected at 17°, and only moderately affected at 37°. Also I showed previously [1913, p. 335] that pancreatic juice activated at 37° has much less activating power on inactive juice than if it is activated at 17°. Deuterase retains its activity almost undiminished for months in glycerin extracts, but is very unstable in aqueous extracts. This is indicated by the small activating power of the HCl and aqueous extracts in the present experiments, and of the 0·9 % NaCl extracts described in my original experiments. Possibly the trypsin itself attacks it. Arguing from the great retardation to the action of deuterase exerted by Witte peptone, it seems probable that pro-deuterase is a protein substance which is first converted into deuterase, and then destroyed, by the digestive action of trypsin.

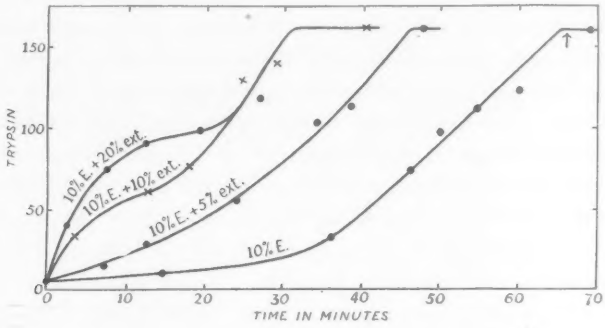

Fig. 15.

THE AUGMENTOR ACTION OF INTESTINAL EXTRACT ON DEUTERASE.

It was stated in an earlier section that when ox-pancreas extract is acted on by enterokinase, it shows the typical acceleration in the velocity of the last half of activation. On an average this latter half took 29 % of the total activation time, so the acceleration was not marked, but the mere fact that it exists at all points to the liberation of deuterase in the extract, or is in apparent contradiction to the result described above. Further experiment showed clearly that there is a formation of deuterase in the course of activation. The addition of 10 % of E. to the extract at 17° gave the curve shown in Fig. 15. Forty-one minutes after the point in this curve marked with an arrow had been reached, 20 % of the activated mixture (containing 2 % of E.) was added to more inactive ox-extract together with 8 % of fresh E. That is to say, the inactive extract was now being activated by 10 % of E., together

with any deuterase which may have been present in 20 % of the previously activated mixture. The curve obtained shows the rapid initial velocity of activation followed by a gradual slowing which is the typical deuterase effect. This is probably followed by a second acceleration of activation, though by an oversight sufficient experimental data were not obtained to prove it, but in another experiment made with 10 % of E. + 10 % of the activated extract 138 minutes after activation, this second quickening of acceleration is shown. Presumably it is dependent on the enterokinase present. In another experiment made 76 minutes after activation, with 10 % of E. + 5 % of the activated mixture, the activation is considerably accelerated, but at a steady rate throughout.

This experiment leaves no doubt that a moderate amount of deuterase must have been liberated from pro-deuterase in the extract of ox-pancreas during the course of its activation by enterokinase, but in such a case how is one to explain the apparent absence of deuterase as shown in the experiments of the type described in the last section ? The subject is a difficult and complex one, and I did not attempt to solve it in detail, but the experiment to be described points to the existence of an anti-body which neutralises the deuterase unless its action is augmented by the presence of enterokinase. Some of the extract of ox-pancreas was activated at 17° by the addition of 5 % of active extract of pig's pancreas, and 8, 56, 128 and 174 minutes after activation was practically complete 5 % samples of this activated extract were added to more inactive extract, together with 5 % of E. In every case activation occurred more rapidly than in the control experiment in which E. alone was added. With the 128 minutes sample it was completed in 81 minutes instead of the 109 minutes taken in the control experiment, and with the other samples in 84 to 90 minutes. In the absence of E. the samples had no activating effect at all.

The augmentor action of intestinal extract upon deuterase was proved by a number of experiments. For instance, Fig. 16 shows the effect upon inactive extract of ox-pancreas at 37° of (*a*) 1·25 % of deuterase (active glycerin extract of monkey's pancreas); (*b*) 1·25 % of deuterase which had been diluted tenfold and kept with 10 % of E. at 17° for 2 minutes before mixing with the inactive ox extract; (*c*) 1·25 % of deuterase kept with the E. for 62 minutes before mixing with the inactive extract. We see that in this last experiment activation occurred considerably quicker than in the first or even in the second experiment. In fact it was almost doubled in rate, as can be seen on comparing the curve with the control

curve obtained with 2·5 % of active extract. Hence it is evident that the trace of E. in some way augments the action of the deuterase, and that to get the best result it is necessary for the deuterase and E. to be mixed for some little time before they are added to the inactive extract. In another experiment, with different extracts, it was found that when the deuterase and E. were mixed for 32 minutes before, they had just the same activating effect as when they were mixed 69 minutes before, activation being in each case completed in 18 minutes instead of 24 minutes, the time taken when they were mixed only 1 minute before. If the time for which they are mixed be too prolonged the activating effect is diminished rather than increased,

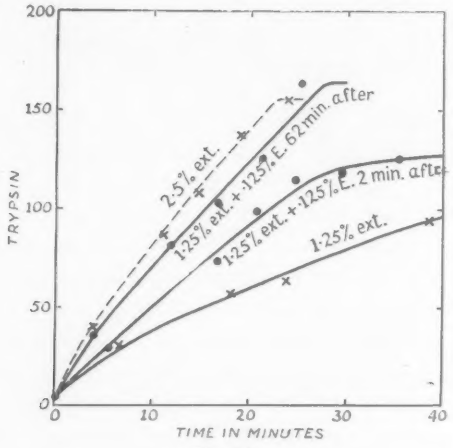

Fig. 16.

as the enzymes appear to destroy one another. Thus in one experiment it was found that if they were mixed 148 minutes before adding to the inactive extract there was no augmentation of activation at all, whilst in experiments in which they had been mixed for 24 hours there was a considerable diminution of activation.

It might be thought that the increased rate of activation was due directly to the E. but this is not so, for the actual amount added in the experiment recorded in Fig. 16, viz., 0·125 %, had by itself no appreciable activating effect in the time for which the experiment lasted. In another experiment the proportion of E. added was large enough to produce a considerable amount of activation. An extract of sheep's pancreas was activated at 37° by (*a*) 2 % of deuterase (active extract of pig's pancreas); (*b*) 0·25 % of E.

(a very active preparation); (*c*) 2 % of deuterase + 0·25 % of E., mixed together in presence of 0·05 % Na_2CO_3 and kept at 37° for 1·5 minutes before adding to the inactive extract. This mixture liberated 43, 104, 164 and 191 units of trypsin after 10, 15, 20 and 25 minutes respectively, whilst the sum of the amounts liberated by the deuterase and the E. after the same time intervals amounted only to 22, 43, 77 and 145 units.

An experiment in which enough E. was taken to activate a glycerin extract of pig's pancreas completely in 60 minutes at 17° is recorded in Fig. 17. This activation is compared with that produced by (*a*) 1 % of deuterase (active extract of pig's pancreas); (*b*) samples of 1 % of deuterase + 0·5 %

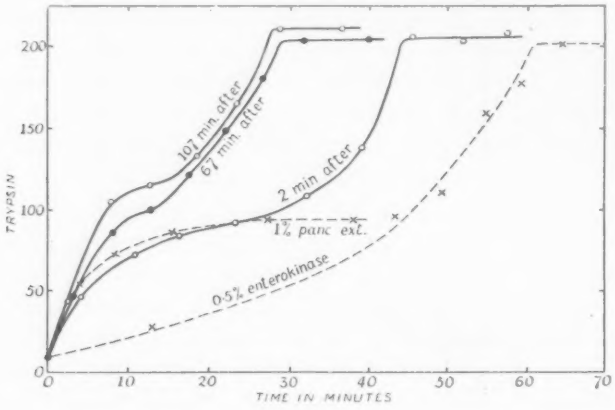

Fig. 17.

of E. mixed together at 17° in presence of 5 volumes of 0·05 % Na_2CO_3, and added to the inactive extract, 2, 67 and 107 minutes later. The curve obtained with the 2 minutes mixture shows a less rapid initial rate of activation than that produced by 1 % of deuterase alone, and much less than the sum of the effects produced by 1 % of deuterase + 0·5 % of E. The 107 minutes mixture, however, yielded an activation curve the earlier part of which would about correspond to the sum of the deuterase and E. curves, and the later part of which represents a distinct augmentation on such effects. The retardation produced when deuterase and E. solutions are mixed in certain proportions and added immediately to inactive extract appears to be greater the larger the proportion of E. used, for in another experiment at 17°, upon inactive sheep's extract, the addition of a mixture of 5 % of deuterase (active sheep's extract) + 10 % of E. (an old and rather weak preparation) caused

activation in 95 minutes when added immediately after mixing, though the 10 % of E., when acting alone, caused activation in 58 minutes. The mixed deuterase + E., when tested 62 minutes after mixing, took 77 minutes to activate; 116 minutes after mixing, 62 minutes to activate; 187 minutes after, 36 minutes, and 229 minutes after, 32 minutes. The 5 % of deuterase, when acting alone, activated three-fourths of the trypsinogen in the first 33 minutes, and then almost stopped, but even the activation induced by the 229 minutes' mixture was only equal to the sum of the activation rates induced by the deuterase alone and the E. alone. Hence in this experiment the large quantity of E. added, or some anti-body contained in the preparation, exerted a retarding action throughout.

General Conclusions.

It is desirable to discuss briefly some of the objections which may be levelled against my hypothesis, and to show in what respects it can be reconciled with previous observations. In the first place it may be said that it is inherently improbable that two different enzymes should be required to bring about one and the same effect. In reply to this I would point out that several different agents are capable of liberating secretin from prosecretin. In addition to acids the liberation is effected by soaps, alcohol, 0·6 % NaCl and other substances [Matsuo, 1913]. These liberating substances cannot all act in the same way; neither can pepsin and trypsin attack proteins in the same way, and so there is no real difficulty in the fact that enterokinase and deuterase attack trypsinogen in different ways. The extremely low temperature coefficient observed for deuterase, viz., 1·12, is unique, but comparatively low coefficients have been observed with other enzymes. Kastle and Loevenhart [1900] obtained one of 1·3 for the action of esterase on ethyl butyrate. Tammann [1892], Kjeldahl [1881], and O'Sullivan and Tompson [1890] independently obtained the same coefficient, viz., 1·4, for the action of invertase on cane sugar, whilst Senter [1903] obtained a coefficient of 1·5 for the action of catalase on hydrogen peroxide. Most other enzymes investigated, including enterokinase, have been found to give coefficients of 2·0 to 2·6 [for literature see Euler, 1910], but Bayliss [1904] observed one of 5·3 for the action of trypsin on caseinogen, and as I [1904, 1, p. 364] observed one of 2·2 to 1·8 for the action of trypsin on Witte peptone one must conclude firstly that different enzymes have very different coefficients from one another, and secondly that even with the same

enzyme the coefficient varies with the nature of the substrate. In this connection it will be remembered that deuterase gave coefficients varying from 1·0 to 1·2 with different substrates.

Bayliss [1911] points out that the temperature coefficient of adsorption is, so far as investigated up to the present, a low one. He himself found that the rate at which equilibrium is attained in the adsorption of Congo red by paper is accelerated 1·36 times for each rise of 10° between 10° and 50°. Presumably the still lower coefficient obtained with deuterase is likewise compatible with the view that its action is an adsorption process, and involves no chemical change such as is associated with the action of enterokinase. The low coefficient of deuterase cannot be taken to indicate that it is not a true enzyme, for we have seen that it acts catalytically according to the logarithmic or the linear law like other enzymes. Moreover it is an extremely unstable body.

As another objection to my hypothesis it may be said: What is the need of a dual mechanism of activation? A convincing answer is supplied by the experiments of Terroine [1910] and of Mellanby and Woolley [1914, 2], who have shown that pancreatic steapsin is extraordinarily unstable in the presence of free trypsin. If enterokinase is added to pancreatic juice, the steapsin persists only so long as the quantities of trypsin present are minute. Hence, as Mellanby and Woolley point out, it is essential that the digestion of fat in the small intestine should take place in the absence of much free trypsin. This condition is observed in the upper part of the small intestine provided that there is not much enterokinase present, for in such a case the rapid liberation of trypsin from the trypsinogen of the juice may be delayed for many minutes.

An objection may be raised to the fact that enterokinase apparently has two distinct actions. We have seen that it not only liberates trypsin from trypsinogen, but it heightens the activity of deuterase on trypsinogen. This fact naturally suggests that deuterase is merely a co-enzyme of enterokinase, but arguments precluding this view have been adduced in a previous section. It may be found that these two properties of enterokinase are in some way related to one another, though at present we cannot say how. Again, it is possible that enterokinase acts by neutralising an anti-body to deuterase which is present to a greater or less extent in all extracts. Moreover, the augmentor effect on deuterase may not be due to enterokinase at all, but to some other unknown substance present in intestinal extracts.

The nature of the change involving the conversion of trypsinogen into trypsin is unknown. Mellanby and Woolley suggest that enterokinase is a proteolytic ferment which activates trypsinogen by digesting it, and splitting off free trypsin from a protein moiety with which it is firmly combined. Some of my earlier experiments [Vernon, 1903] show this hypothesis to be very improbable. I determined the proportions of trypsinogen precipitated from a glycerin extract by the addition of various strengths of alcohol, and on repeating the experiment a few days later when the trypsinogen had been converted into trypsinogen I found the precipitability of this trypsin to be almost exactly the same. It might be argued that the alcohol really precipitated protein from the extract, which absorbed and carried down trypsinogen or trypsin with it, but in reply I would point out that I found the precipitability of another enzyme in the extract, viz. amylopsin, to be quite different from that of the trypsin. Hence it is highly improbable that a protein + trypsin body would be adsorbed exactly to the same extent as trypsin alone. In all probability the conversion of trypsinogen into trypsin is a transformation involving no change in the size of the molecule, and this can be induced either by a mechanism which involves a chemical process at some stage, or by a purely adsorption process, *i.e.*, by enterokinase or by deuterase.

If the results described in this paper be compared with those given previously [Vernon, 1913], it might be thought that they are to some extent mutually contradictory. Thus I showed that inactive juice could readily be activated by the addition of activated juice; whether this contained enterokinase or not, but the activation curves which I gave do not, except in one experiment, show the initial rapid activation which is typical of the action of deuterase. The explanation is that I did not as a rule happen to catch my samples of activated juice at a time when they still retained much of their unstable deuterase, and that in consequence the curves given in Figs. 2 and 4, and the curve on the right of Fig. 3 of my previous paper, are comparable to the lowest curve in Fig. 9 of the present paper, whilst the curve on the left of Fig. 3 of my previous paper is comparable to that on the extreme left of Fig. 9 of the present paper. Again, Mellanby and Woolley [1913, p. 355] quote an experiment in which they activated juice by enterokinase, and every few minutes during the course of activation they took 10 % samples of it and added them to inactive juice. On plotting out their results it can be calculated that the sample taken when the primary activation was just completed had nearly twice the activating power of the sample

taken when it was just begun, and yet they do not admit that this result affords proof of the existence of any activating agent other than enterokinase. As is indicated by the results described in this paper, they would have found that their activated juice had a much greater activating power if they had tried it a few minutes after it had completed its activation, and still better, if they had carried out the experiment at 17° instead of 37°.

Summary.

1. The experiments described in this paper lead to the conclusion that in the activation of trypsinogen by enterokinase, the trypsin liberated in the earlier stages by the direct action of the enterokinase gradually sets free an enzyme (termed *deuterase*, to indicate that it acts secondarily to enterokinase) from a precursor, and that this deuterase is mainly responsible for the later stages of the activation process.

2. In support of this hypothesis it is found that in the activation of pancreatic juice by enterokinase, the rate of activation during the last half of the process may be 1000 times more rapid than the initial rate, and it is independent of the quantity of enterokinase used. The total activation time varies with the temperature (temp. coefficient for 10° = 2·00), but the time taken over the last half of activation is only slightly influenced by temperature. This is because it is chiefly dependent on deuterase, the temperature coefficient of which ranges from 1·00 to 1·22. Enterokinase and deuterase are also distinguished by the fact that the former is relatively much more retarded in its action by glycerin, and the latter, by Witte peptone.

3. The addition of active pancreatic extract or juice to inactive extract or juice causes activation which is rapid at first, but which quickly slows down almost to zero. This is due to its containing dueterase, which acts according to a logarithmic or linear law like other enzymes. The freshly activated extract or juice contains very little deuterase, but it is gradually liberated from pro-deuterase in the course of the next few minutes (at 37°), or next hour or two (at 17°). The rate of its liberation increases in geometrical progression.

4. There is no relationship between the amounts of deuterase and trypsin present in pancreatic extracts prepared from various animals. Glycerin extract of monkey's pancreas contained only a fourth as much trypsin as glycerin extract of pig's pancreas, but two-thirds as much deuterase. Some experiments seemed to show that extract of ox pancreas contained no deuterase

at all, but this was because its presence was masked by an anti-body. Deuterase is extremely unstable, except in presence of glycerin, and therefore aqueous pancreatic extracts have relatively little activating power, whilst freshly activated pancreatic juice was observed to lose most of its activating power in two hours at 37°. Probably the deuterase is destroyed by the trypsin.

5. The stability of the trypsin in different extracts is extremely variable, and bears no relationship to activating power. That in ox pancreas extract was destroyed at the rate of 78 % per hour by 0·05 % Na_2CO_3 at 37°, whilst that in pig's pancreas extract was destroyed at 12 % per hour. Trypsinogen also exists in different degrees of stability, and in one extract half of it was found to be activated by enterokinase seven times more slowly than the other half.

6. The activating power of deuterase may be nearly doubled by the addition of small quantities of intestinal extract which themselves have no appreciable activating power. Nevertheless deuterase is not a co-enzyme of enterokinase.

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LV. THE INFLUENCE OF EXCESSIVE WATER INGESTION ON PROTEIN METABOLISM.

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A number of observations have been made upon the effects of increased ingestion of water on the output of nitrogen. As the various results obtained by previous workers are referred to later, it will suffice here to state that there is almost unanimous agreement that if water be freely exhibited to an animal in nitrogenous equilibrium there accompanies the consequent diuresis an increased excretion of nitrogen. As to whether this increased nitrogenous excretion signifies a mere mechanical flushing out of waste products or an accelerated catabolism of protein, opinion has been sharply divided. Fowler and Hawk [1910] undertook an investigation on the subject and extended the field of observation to include creatine and creatinine. They found regarding these, that the addition of three litres of water per day produced a decrease in the output of creatinine and led to the appearance in the urine of considerable amounts of creatine.

The primary object of the present research was to investigate the accuracy of these findings. In view of the fact, however, that most of the earlier workers conducted their experiments on animals and limited their observations either to the total nitrogen or the urea, it was thought that further information might be obtained by making a more complete urinary analysis in the human subject and by varying the protein intake in the different experiments.

Method.

The subjects of the experiments, normal healthy males, were given a fixed creatine-free diet for several days, until the daily excretion of the various nitrogenous constituents of the urine had become approximately constant.

Thereupon, in addition to the diet, a given quantity of water was drunk each day for three days, whereafter, the diet was continued for several days more without the extra water. Some of the water was taken with the meals and the rest in the intervals between. The protein content of the food was varied in the different experiments from 27 to 319 grams per day. The urine was collected in 24 hour samples, the collection being completed at 9 a.m. before either food or water had been taken for the day.

In one of the experiments, No. IV, the water taken during the preliminary period was fixed in amount. The daily requirements however, as indicated by the inclination of the subject to drink, were not uniform. In all the others therefore, to ensure that the conditions on the pre-water days would be as natural as possible, the amount taken was not restricted. It was considered that this procedure would give a more perfectly normal period for comparison with the period of excessive consumption, and that the volume of the urine would indicate the average daily intake, with sufficient accuracy for the purposes of the research.

The methods of analysis adopted were: total nitrogen—Kjeldahl: ammonia—Folin: urea Exp. II, III and IV,—Folin, I and V,—the urease method described by Plimmer and Skelton [1914]: amino acids—Sörensen's formalin titration method: creatine and creatinine—Folin.

Protocols.

Experiment I. Very low protein diet.

Diet. In grams, apple 100, banana 150, potato 400, butter 75, sugar 75, cocoa 10, bread 200, containing approximately protein 27 g., fat 70 g., carbohydrate 325 g.

Subject aet. 33; weight at beginning of exp. 67·8, at end 65·8 kilos.

The diet was continued for a preliminary period of eight days. On the 9th, 10th and 11th days three litres extra of water were drunk. Owing to the rapid loss of weight of the subject the experiment was stopped on the 12th day.

The results of the first six days are omitted, the only points of interest being a continuous loss of weight, an excretion of nitrogen in excess of the intake and a uniform decrease of creatinine from 1·367 to 1·320 grams per day.

TABLE I.

Day of exper.	Weight kilos	Urine c.c.	Tot. N. grs.	Urea N. grs.	NH_3 N. grs.	Amino Acid N. grs.	Creatinine grs.	Creatine grs.	Per cent. of Tot. N. as urea	Remarks
7	65·97	412	6·300	4·151	0·259	0·113	1·320	—	65·9	Fixed diet
8	65·97	405	6·300	4·157	0·185	0·143	1·328	—	65·9	,,
9	65·80	2830	8·610	6·629	0·231	0·163	1·330	—	76·9	3 lit. H_2O extra
10	66·02	3545	6·325	4·534	0·266	0·161	1·315	—	71·6	,,
11	65·80	3602	6·125	4·388	0·280	0·108	1·313	—	71·6	,,

Experiment II. Moderately low protein diet.

Diet. In grams, oatmeal 100, bread 300, butter 80, apple 70, cocoa 10, sugar 100, milk 300 c.c., containing approximately protein 48 g., fat 93 g., carbohydrate 346.

Subject aet. 22; weight at beginning of exp. 57·8, at end 57·7 kilos.

The diet was continued for a preliminary period of seven days. On the 8th, 9th and 10th days three litres extra of water were drunk. On the 11th, 12th and 13th days the diet was continued without extra water. The first five days are omitted from the table, as they show no points of interest. The first post-water day is also omitted, as the analysis showed the day's collection to have been contaminated.

TABLE II.

Day	Body wt. kilos.	Urine c.c.	Tot. N. grs.	Urea N. grs.	NH_3 N. grs.	Amino Acid N. grs.	Creatinine grs.	Creatine grs.	Per cent. of Tot N. as urea	Remarks
6	57·7	600	9·07	6·10	0·342	0·1834	1·350	—	67·3	Fixed diet
7	57·7	670	8·77	5·95	0·347	0·1788	1·421	—	67·8	,,
8	58·4	2980	9·83	6·90	0·459	0·3633	1·360	—	70·2	3 lit. H_2O extra
9	58·2	3105	8·96	6·12	0·465	0·1827	1·360	—	68·3	,,
10	58·2	3400	9·24	6·41	0·459	0·1970	1·377	—	69·4	,,
12	57·7	880	9·38	6·44	0·336	0·1970	1·343	—	67·6	Fixed diet
13	57·7	870	9·46	6·59	0·403	0·1480	1·350	—	69·7	,,

Experiment III. Moderately high protein diet.

Diet. In grams, sugar 100, "plasmon" 60, cheese 200, dried skimmed milk 50, bread 400, cocoa 20, butter 50, apple 100, containing approximately protein 160 g., fat 73 g., carbohydrate 359 g.

Subject same as Exp. II, aet. 22; weight at beginning of exp. 57·2, at end 57·4 kilos.

On the 5th, 6th and 7th days three litres extra of water were drunk. On the third post-water day the diet was stopped. The table gives the results from the third day.

TABLE III.

Day	Body wt. kilos	Urine c.c.	Tot. N. grs.	Urea N. grs.	NH_3 N. grs.	Amino Acid N. grs.	Creatinine grs.	Creatine	Per cent. of Tot. N. as urea	Remarks
3	57·2	1220	17·53	13·98	0·468	0·1274	1·377	—	79·7	Fixed diet
4	57·3	1040	17·47	14·03	0·392	0·1060	1·381	—	80·3	,,
5	58·8	3450	19·04	14·54	0·609	0·1000	1·332	—	76·4	3 lit. H_2O extra
6	58·8	3895	19·06	15·87	0·566	0·1081	1·405	—	83·3	,,
7	58·9	2730	18·72	14·99	0·549	0·1172	1·343	—	80·1	,,
8	57·6	1350	19·49	16·26	0·426	0·2744	1·400	—	83·4	Fixed diet
9	57·4	1220	20·12	16·67	0·440	0·0792	1·340	—	82·9	,,

Experiment IV. Very high protein diet.

Diet. In grams, dried skimmed milk 600, cheese 300, bread 300, water 3 litres containing approximately protein 319 g., fat 153 g., carbohydrate 179 g.

Subject aet. 21; weight at end of exp. 57 kilos.

In this experiment the diet was continued for 15 days. On the 5th and on the 9th, 10th and 11th days 9·6 litres of water were drunk in addition to the water used in preparing the food.

TABLE IV.

Day	Urine c.c.	Tot. N. grs.	Urea N. grs.	NH_3 N. grs.	Amino Acid N. grs.	Creatinine grs.	Creatine grs.	Per cent. of Tot. N. as urea	Remarks
1	1930	39·82	33·59	0·250	0·241	1·660	—	84·4	Fixed diet
2	1980	40·24	33·83	0·241	0·249	1·480	—	84·1	,,
3	2180	42·19	33·90	0·246	0·264	1·563	—	80·4	,,
4	2260	43·88	35·03	0·206	0·324	1·554	—	79·8	,,
5	7400	40·19	36·41	0·477	0·103	1·533	—	90·6	9·6 litres H_2O
6	2150	34·60	31·71	0·202	0·348	1·588	—	91·4	Fixed diet
7	2110	38·19	35·36	0·283	0·267	1·650	—	92·6	,,
8	2040	40·22	36·58	0·299	0·398	1·604	—	91·0	,,
9	8370	40·19	37·05	0·576	0·268	1·586	—	92·2	9·6 litres H_2O
10	8500	40·57	36·68	0·563	0·436	1·459	—	90·4	,,
11	8070	39·24	36·12	0·499	0·387	1·547	—	92·1	,,
12	2440	34·16	31·28	0·252	0·348	1·687	—	91·6	Fixed diet
13	2150	36·34	33·46	0·333	0·279	1·605	—	92·1	,,
14	2160	36·89	33·96	0·345	0·285	1·619	—	92·1	,,
15	2300	40·40	37·18	0·361	0·363	1·642	—	92·0	,,

Experiment V.

Diet creatine-free, containing protein 110, fat 67, carbohydrate 325 g.

In this experiment nitrogenous equilibrium had not been attained before the increased water intake. Its results are omitted in discussing the effects of water on the excretion of total nitrogen.

TABLE V.

Urine c.c.	Tot. N. grs.	Urea N. grs.	NH_3 N. grs.	Amino Acid N. grs.	Creatinine grs.	Creatine grs.	Per cent. of Tot. N. grs.	Remarks
1298	10·885	8·458	0·322	0·243	1·330	—	77·7	Fixed diet
898	12·810	9·940	0·280	0·261	1·340	0·02	77·6	,,
3741	13·583	10·962	0·378	0·257	1·340	0·02	80·7	3 litres H_2O extra
4020	13·02	10·596	0·406	0·229	1·385	—	81·4	

Results.

Total Nitrogen. In all except Exp. IV the increased ingestion of water causes a distinct rise in the excretion of total nitrogen. There is, however, in the degree of the increase a lack of uniformity, which corresponds to the divergent results obtained by different workers. Thus Forster [1878] found an increase of 90 %, Heilner [1906] 40 %, Voit [1860] 25 %, Mayer [1880] 9 %, Gruber [1901] 7 %, Fränkel [1877] 6–12 %, and Salkowski and Munk [1877] about 3 %. Dubelir [1891], Seegen [1871] and Straub [1899] found no distinct increase. Heilner [1906] suggests that the results vary with the amount of food. Voit and Forster worked with fasting dogs, while Dubelir and others used dogs on an ample diet of flesh. In the present series of experiments the greatest increase was obtained in Exp. I, where the protein was abnormally low, whereas in Exp. IV with the high protein intake there was no increase. In Table VI the whole series is compared.

TABLE VI.

No. of exper.	Protein in diet. approx. g.	Excretion of Tot. N. on last pre-water day, g.	Excretion of Tot. N. on first water day, g.	Increase in Tot. N. g.	Increase per cent.
1	27	6·30	8·6	2·3	36·5
2	48	8·77	9·83	1·06	12·1
3	160	17·47	19·04	1·57	8·9
4	319	40·22	40·19	*nil*	*nil*

It is thus seen that the percentage increase of total nitrogen excreted on excessive water ingestion tends to vary inversely with the amount of protein fed.

In Exp. IV there is evidence of a marked retention of nitrogen on the cessation of the excessive water intake. After the single water-drinking day the amount excreted fell from 40·19 to 34·69 grams. After the three-day period of increased water intake it fell from 39·24 to 34·16, and only returned to its former level on the fourth day.

There would appear to be two distinct factors involved, one, whose action is immediate and whose influence is most manifest in protein deficiency, tending to cause a flushing out of nitrogenous end products, and another, whose influence is more prolonged, tending to cause a retention of nitrogen.

Ammonia. There is in every instance a distinct rise in the ammonia output which coincides exactly with the increased water intake. This is in keeping with the results of Fowler and Hawk [1910], who argued that the water stimulated gastric secretion and the increased ammonia represented the amount necessary to neutralise the excess of HCl thereby produced. If this argument were well founded the increase in ammonia output should be more or less parallel with the increase in water drunk. As appears in Table VII, there is no evidence of such parallelism.

The increase of water passing through the system must accelerate the flow of blood through the liver and of lymph through the tissues. In the process of deaminisation, as the NH_2 moiety is detached it would tend to be carried off by the accelerated flow of blood and lymph and excreted as ammonia instead of being converted to urea. If this supposition be correct, the increase in ammonia would be in some degree proportional to the extent of deaminisation taking place in the tissues, which in turn would be influenced by the amount of protein in the food. The following table shows that the increase in ammonia more or less corresponds to the amount of food protein being catabolised.

TABLE VII.

No. of exper.	Protein in diet, g.	NH_3N. Aver. excret. on non-water days, g.	NH_3N. Aver. excret. on water days, g.	Increase g.	Percentage increase
1	27	0·222	0·259	0·037	16·6
2	48	0·345	0·461	0·116	33·6
5	110	0·301	0·392	0·091	30·2
3	160	0·430	0·575	0·145	33·7
4	319	0·273	0·529	0·256	93·8

The increase in ammonia evidently represents a mere flushing-out process, and the amount of the increase depends upon the amount of ammonia being liberated in the liver and other tissues.

Urea. The rise in the urea output is most marked. Both in absolute amount and in percentage of total nitrogen excreted as urea, the increase is maintained throughout the water and the post-water periods. Thus, in Exp. I. on the third water day the total nitrogen falls from 6·3 grams on the pre-water days to 6·125, while the urea rises from 4·154 to 4·388. In Exp. II

the average urea output of the post-water days is 6·52 as against 6·03 on the pre-days. In Exp. III the figures are: pre-water 14 g., post-water 16·46 g. In Exp. IV, on the last day of the records, i.e. the fourth day after the period of increased water intake, the total nitrogen output is 40 g. as compared with an average of 41·53 g. in the preliminary period, while the urea, instead of decreasing with the total nitrogen, rises from 34·09 to 37·184 g. The prolongation of the increased excretion of urea beyond the period of increased diuresis is evidence that the increase cannot be accounted for by a mere mechanical flushing out of urea accumulated in the tissues.

Table VIII shows the percentage of total nitrogen excreted as urea in the preliminary period and in the period following the beginning of the increased consumption of water including both the water and the post-water days.

TABLE VIII.

	Average daily excretion in preliminary period			Average daily excretion on water and post-water days			
No. of exper.	Tot. N. g.	Urea g.	Per cent. of Tot. N. as urea	Tot. N. g.	Urea g.	Per cent. of Tot. N. as urea	Increase per cent.
1	6·3	4·154	65·9	7·02	5·184	73·8	7·9
2	8·92	6·025	67·5	9·374	6·492	69·3	1·8
3	17·50	14·005	80·0	19·286	15·666	81·2	1·2
	41·532	34·09	82·8	38·27	35·07	91·6	8·8
5	11·848	9·199	77·6	13·30	10·779	81·0	3·4

It will be seen that the greatest increase in the excretion of urea occurs in Exp. I, where there is a protein deficiency, and in Exp. IV, where the protein intake is excessive.

Amino acids. In Exps. I and II on the low protein diets there is an immediate rise in the excretion of amino acids on the first day of increased water ingestion. In Exp. III with a moderately high protein intake there is a slight drop, and in Exp. IV, where the protein intake is higher, the drop is marked. Table IX compares the series.

TABLE IX.

No. of exper.	Protein intake, g.	Amino acid N. Average in prelim. period	Amino acid N. First water day	Difference
1	26	0·128	0·163	+·035
2	48	0·181	0·363	+·182
5	110	0·255	0·257	+·002
3	160	0·117	0·100	−·017
4	319	0·270	0·103	−·168

While there is an absence of uniformity in the results, they are such as suggest the condition, that with a deficiency of protein in the food the stimulated diuresis due to increased water intake causes an initial washing out of the amino acids, while with an excess of protein the increased water intake causes an initial retention of these. As these results are not maintained, they would appear to be due to the sudden alteration in the amount of fluid passing through the system.

Faecal nitrogen. Only in Exp. I was the collection and analysis of faeces made. The daily evacuation was fairly regular, taking place in the forenoon. The first day's collection was discarded, as they belonged more properly to the preceding period. Those of the first water day were regarded as belonging to the preliminary period and those of the first post-water day as belonging to the water period. The results obtained were :

Preliminary period		Increased water period	
Dried weight	Total nitrogen	Dried weight	Total nitrogen
19·42 g.	0·927 g.	12·9 g.	0·542 g.

These results are in agreement with those of Fowler and Hawk [1910]. Too much importance should not be attached to the results of a single experiment, but even keeping in view the fact that the faecal nitrogen is not all derived from food residues, the results seem to suggest that the increased consumption of water is productive of a more complete utilisation of the ingested protein.

Creatine and creatinine. In Exps. II, III and IV there is on the water days an apparent decrease in the excretion of creatinine. On these days, however, the bulk of the urine was enormously increased and the concentration of the creatinine correspondingly decreased. It was necessary, therefore, to depart from the routine mode of analysis on the water days. In some instances 30 c.c. of urine were taken for analysis instead of 10 c.c., and in other instances, after development of the colour, dilution was carried only to 250 c.c. instead of to 500 c.c. Both of these deviations, which are recommended by Folin [1904] in such cases, in his original description of the colorimetric method are productive of error, showing a reduction in the amount of creatinine found which is quite fictitious. In Exps. I and V the urine from the beginning was diluted to 5,000 c.c. and the dilution and method of analysis rigidly adhered to throughout the experiment. In these, on the water days there appears no decrease in the amount of creatinine excreted. It may be concluded, therefore, that the decrease found in the earlier experiments should be ascribed to faulty methods of analysis. On no occasion was the increased

consumption of water followed by the appearance of creatine in the urine. The influence of excessive water ingestion on the excretion of creatine and creatinine has already been dealt with [Burns and Orr, 1914] and need not be further discussed here.

Discussion of Results.

The source of the increased excretion of nitrogen on excessive consumption of water has been much discussed. V. Noorden [1907], after a review of the literature, says: "It all turns on the flushing of nitrogenous end products out of the system." He bases his conclusion largely on the work of Neumann [1899], who found that, as the consumption of water rose, the urinary nitrogen rose, the rise being most marked on the first day and rapidly disappearing, and that on a return to a normal consumption of water there occurred a retention of nitrogen. The initial increased output and subsequent retention have been here verified, but the compared results obtained on the varying protein intakes do not support the view that the increase represents a mere flushing out of end products. If that view were correct, the greatest increase would appear where there existed the greatest amount of end products, which one would expect to be during the period of highest protein intake. We have seen however that it is just in this case that no increase takes place, a result which is in agreement with the findings of those who worked with dogs on a heavy flesh diet, and that, on the contrary, the highest increase is found in Exp. I, where the protein was abnormally low, viz. 27 grams per day, the diet consisting mostly of carbohydrate and fat, in which case one would expect the minimum accumulation of nitrogenous end products liable to be flushed out. While it is not to be doubted that there occurs a flushing-out process, to which the increase of ammonia appears entirely due, the whole of the influence exerted by the water cannot be ascribed to this mechanical cause. The results obtained on the varying protein intakes, together with the fact that the increased percentage of urea continues on the post-water days, after the mechanical flushing-out process has ceased, indicate that the water has a direct influence on the protein metabolism.

Voit [1860] believed that the influence of the water is to produce an increased protein catabolism, a view supported by Forster [1878] and others. In conditions of protein deficiency as in fasting dogs with which Voit worked, such a conclusion appears reasonable. In Exp. IV, however, where the protein of the food is excessive in amount, there appears on the addition of

the extra water no increase in the urinary nitrogen, but there occurs a most marked rise both in the absolute amount of urea and in the percentage of total nitrogen excreted as urea, a result obtained in all the experiments. While it is doubtful, therefore, whether in conditions of protein sufficiency excessive consumption of water causes an increase in the amount of protein catabolised, as indicated by the urinary nitrogen, there appears to result in every case a stimulation of the catabolic processes, leading to a more complete disintegration of the protein molecule and the production of those end products whose immediate destination is excretion.

The subsequent marked retention of nitrogen in Exp. IV is in agreement with the results obtained by Neumann [1899] and Fowler and Hawk [1910]. In view of the fact that the retention takes place on the highest protein diet (319 grams per day), when there would be a surplus of circulating protein and non-protein nitrogenous material within the economy, it is improbable that the amount retained, viz. about 8 grams after the one day's water drinking, and in addition to that about 14 grams after the three day's period, would represent an addition to either of these bodies. It is more probable that the retained nitrogen represents an increase in tissue protein. If this supposition be regarded as well founded, we would be able to conclude that the influence of the water is to stimulate both the analytic and the synthetic phases of protein metabolism.

An observation made on the body temperature in Exp. I lends support to this view. During the preliminary period the average temperature was 97·1° F., and during the water days the average was 97·6° F. Heilner [1907] observed an increased production of heat in rabbits on increased water ingestion.

Summary.

The excessive ingestion of water produces :

1. An increased excretion of urinary nitrogen which is most marked on a low protein diet.

2. A retention of nitrogen on the return to normal consumption of water in the case of excessive protein intake.

3. An increase in the percentage of total nitrogen excreted as urea.

4. A marked increase in the excretion of ammonia.

5. No excretion of creatine and no decrease in the excretion of creatinine.

6. A decrease in the faecal nitrogen which is interpreted as indicating a more complete utilisation of the food protein.

It is suggested that the results indicate that the influence of the increased water consumption is to accelerate both the catabolic and the anabolic phases of protein metabolism.

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LVI. THE DETERMINATION OF THE COMPOSITION OF THE DIFFERENT PROTEINS OF OX AND HORSE SERUM BY THE METHOD OF VAN SLYKE.

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Recent investigations have shown that serum albumin and serum globulin exhibit well marked differences in chemical composition. Abderhalden [1903, 1905] working with the proteins of horse serum found that albumin yielded no glycocoll on hydrolysis, while globulin gave 3·5 % of this amino acid. Haussmann [1899] and Gümbel [1904] studied the albumin of horse serum by Haussmann's nitrogen distribution method and Gibson [1912] using the same method determined the nitrogen distribution in the pseudo-globulin from horse serum. The results obtained by these three authors show that albumin contains a greater proportion of basic or diamino nitrogen than globulin. Bywaters and Tasker [1913] determined the sulphur and carbohydrate content of the albumin and globulin of horse serum. They found that albumin contains about 2 % of sulphur and 0·25 % of carbohydrate, while globulin yields less sulphur (1·17 %) and more carbohydrate (3·23 %). They also demonstrated the presence of phosphorus in globulin but could find none in albumin.

The globulin occurring in serum is of two kinds, viz. euglobulin and pseudo-globulin. The physical properties and solubilities of these two substances are very different, and recently evidence has been brought forward to show that they differ also in chemical composition. Hardy [1905] was the first to show that globulin contained phosphorus. He was unable to prepare a phosphorus-free globulin by extraction with alcohol and ether and concluded that the phosphorus of globulin is not due to entangled lecithin. Hardy also

succeeded in preparing an osazone from the insoluble phosphorus-containing globulin but failed to do so from the soluble phosphorus-free globulin. Haslam [1912, 1913] prepared pseudo-globulin free from phosphorus and showed that euglobulin contains about 0·1 % of phosphorus, about half of which he was able to extract by means of alcohol and ether. Hardy and Haslam worked with the proteins of ox serum.

In the experiments described in this paper the different proteins of ox and horse serum have been prepared and their chemical composition has been determined by the method of protein analysis recently introduced by van Slyke [1911]. This method has the advantages of yielding approximately quantitative results and requiring the use of relatively small quantities of material, and indicates the nature of the nitrogenous products resulting from complete acid hydrolysis. The whole proteins of the serum, the albumin, the total globulin, the pseudo-globulin and the euglobulin have been prepared in as pure a condition as possible. Each protein was finally obtained in the dry condition and weighed quantities of the air-dried preparations were analysed. Duplicate analyses were carried out in all cases in which sufficient material was available. Details of the preparation of the various proteins and the figures obtained in the analyses of the same are recorded at the end of the paper.

Results.

The proteins of ox serum were studied first. Unfortunately, the amount of pure protein available was not sufficient in any case for duplicate analyses to be carried out, and in one case (viz. the euglobulin prepared from ox serum by one-third saturation with ammonium sulphate) only 2·5 g. of material were used. After hydrolysis the solution employed for analysis in this instance contained 235 milligrams of nitrogen: of this, only 52 milligrams were present as diamino nitrogen and the determination of the four diamino acids was carried out on this small quantity. On account of the small amount of material used, less confidence is felt in the result of this particular analysis than in the others. It has been found by experience that the analysis of proteins by van Slyke's method is conveniently carried out on solutions containing about 400 milligrams of nitrogen; in the case of the serum proteins such solutions contain about 100 milligrams of diamino nitrogen. In the case of the proteins of horse serum larger quantities of material were prepared and in three cases enough material was available for duplicate analyses to be carried out.

The results of the analysis of the different proteins of ox and horse serum are summarised in the following tables:

TABLE I.

Summary of the analyses of the different proteins of ox serum, in percentages of the total nitrogen.

	Whole Protein		Albumin		Total Globulin		Pseudo-Globulin		Euglobulin ($(NH_4)_2SO_4$ method)		Euglobulin (Panum's method)	
Ammonia N ..	7·05		5·8		7·7		7·5		9·3		8·0	
Melanin N ..	1·60		1·1		2·0		1·9		2·0		2·5	
Cystine N ..	2·75		3·5		2·0		1·9		2·0		1·4	
Arginine N ..	11·05	31·75	10·4	36·9	10·9	28·2	10·8	27·1	11·6	26·6	11·7	28·7
Histidine N ..	4·40		6·7		6·3		4·8		3·8		6·5	
Lysine N ..	13·55		16·3		9·0		9·6		9·2		9·1	
Amino N of the Filtrate	56·65	58·80	54·2	56·5	59·8	62·0	61·7	63·3	57·9	60·7	58·0	59·4
Non-amino N of the Filtrate	2·15		2·3		2·2		1·6		2·8		1·4	
Sum	99·20		100·3		99·9		99·8		98·6		98·6	

TABLE II.

Summary of the analyses of the different proteins of horse serum, in percentages of the total nitrogen.

	Whole Protein		Albumin		Total Globulin		Pseudo-Globulin		Euglobulin ($(NH_4)_2SO_4$ method)		Euglobulin (Panum's method)	
Ammonia N ..	7·25		6·65		7·95		7·7		7·90		8·0	
Melanin N ..	1·60		0·95		2·30		2·2		2·25		2·3	
Cystine N ..	2·10		3·10		1·65		1·7		1·65		1·8	
Arginine N ..	9·50	30·00	9·90	34·75	8·00	25·25	8·9	26·2	8·25	25·30	9·4	26·5
Histidine N ..	5·60		5·85		4·80		5·8		5·45		5·2	
Lysine N ..	12·80		15·90		10·80		9·8		9·95		10·1	
Amino N of the Filtrate	59·95	61·95	56·55	58·15	62·65	64·80	61·3	64·2	62·20	64·35	61·0	62·8
Non-amino N of the Filtrate	2·00		1·60		2·15		2·9		2·15		1·8	
Sum	100·80		100·50		100·30		100·3		99·80		99·6	

Discussion of Results.

From a consideration of the figures given in the two preceding tables it is evident that the composition of serum albumin is very different from that of any of the globulins. Albumin yields on hydrolysis a greater proportion of diamino acids than the globulins, a result which is in agreement with those obtained by Haussmann [1899], Gümbel [1904] and Gibson [1912], who used the nitrogen distribution method devised by Haussmann. Further, albumin

yields a greater proportion of cystine than globulin. In the case of ox serum albumin 3·5 % of the total nitrogen occurs as cystine, while the average for the four globulins is 1·8 %. The corresponding figures for the albumin and globulin from horse serum are 3·1 % and 1·7 %. The results for cystine are in agreement with those of Abderhalden [1903, 1905], who obtained a greater amount of cystine from albumin than from globulin, and of Bywaters and Tasker [1913], who found that the sulphur content of albumin is greater than that of globulin. Estimations of the lysine, arginine, and histidine content of the serum proteins have not hitherto been carried out. From the figures given above for these amino acids it is clear that in the case of both sera examined the albumin differs from the globulins in containing a much larger amount of lysine. It is also to be observed that albumin yields a smaller amount of ammonia and melanin on hydrolysis than the globulins.

Other experimental evidence regarding the lysine content of albumin and globulin has been obtained. Van Slyke and Birchard [1914] determined the free amino nitrogen of a number of proteins and showed that on treatment with nitrous acid in van Slyke's apparatus for half an hour the volume of nitrogen gas evolved corresponded to one half the lysine nitrogen of the protein. Similar experiments have been carried out with the albumin and globulin prepared from ox serum. It was found that albumin yielded about twice as much nitrogen as globulin on treatment with nitrous acid and that in each case the nitrogen obtained was almost exactly one half the lysine nitrogen of the respective proteins. Full details of these experiments will be published shortly.

The marked difference in composition of the proteins of the serum is of interest when considered with reference to the question of the conversion of albumin into globulin, a change which according to Moll [1904, 1906] occurs when serum is heated for an hour at 60° after the addition of a little dilute alkali. Gibson [1912] and Bywaters and Tasker [1913] brought forward evidence which showed that albumin solutions treated in the manner described by Moll yielded a protein which is not identical with naturally occurring globulin and Abderhalden [1903, 1904] showed that globulin yields glycocoll on hydrolysis while albumin does not. The figures given in the above tables show other important differences in the chemical composition of albumin and the globulins of serum, particularly a striking difference in the relative amounts of lysine. When the whole of the evidence dealing with the chemical nature of the serum proteins is considered it is not easy to understand the mechanism of the process by which such simple treatment

of one protein can lead to the formation of another possessing such a widely different chemical composition.

The figures obtained in the analyses of the different globulins indicate that these substances are very similar in chemical composition. Euglobulin was prepared by two methods: (1) by the method of Panum [1851], and (2) from the previously prepared and purified "total globulin[1]" by one-third saturation with ammonium sulphate. A portion of this "total globulin" was set aside for analysis and the remainder was separated into pseudo-globulin and euglobulin by repeated salting out with ammonium sulphate. Analysis of these different globulin preparations by the method of van Slyke failed to reveal any notable differences in chemical composition. The agreement is particularly close in the case of the globulins prepared from horse serum. In the case of the ox serum globulins, the euglobulin prepared by one-third saturation with ammonium sulphate shows a slightly higher ammonia and lower histidine nitrogen value than the other three, but in other respects the agreement is fairly close. As pointed out above, the analytical errors in this particular estimation are probably greater than in the other cases as the analysis was carried out on a much smaller quantity of material. Larger quantities of this protein were prepared from horse serum and the figures obtained on analysis are almost identical with those obtained for the pseudo-globulin and the "total globulin."

The compositions of the different globulins are of interest when considered in connection with the recent work of Chick [1914]. She suggests that the relationship between euglobulin and pseudo-globulin is a very close one, and that under certain conditions pseudo-globulin may undergo a process of "denaturation" whereby a substance is formed the properties of which are very similar to those of euglobulin. She suggests that this artificial euglobulin may be a mechanical complex resulting from the interaction and mutual precipitation of two colloidal systems (viz. pseudo-globulin solution and lipoid emulsion) and that the euglobulin of serum may be a protein-lipoid complex of similar origin. The evidence brought forward in this paper regarding the composition of these two proteins lends support to the idea that euglobulin may be related to pseudo-globulin in some such way as Chick suggests. The conversion of pseudo-globulin into euglobulin is a process which, on chemical grounds, would be more easily understood and more capable of explanation than the conversion of albumin into globulin.

[1] The term "total globulin" refers to the protein precipitated from the diluted serum by half saturation with ammonium sulphate. It consists of pseudo-globulin and euglobulin.

Experimental Details.

Preparation of the different serum proteins.

Euglobulin was prepared by the method of Panum [1851], and also by means of ammonium sulphate. Albumin, total globulin, and pseudo-globulin were prepared by salting out with ammonium sulphate. The albumin and globulin were first separated by half saturation with ammonium sulphate. The serum was diluted with four volumes of distilled water and an equal volume of saturated ammonium sulphate solution added. The precipitated globulin was centrifuged off and the solution containing the albumin was saturated with powdered ammonium sulphate. The albumin was filtered off, dissolved in distilled water, and the albumin solution brought to a volume equal to that of the original *diluted* serum. An equal volume of saturated ammonium sulphate solution was added, the precipitated globulin was filtered off, and the solution containing the albumin was again saturated with powdered ammonium sulphate. The albumin was dissolved and reprecipitated five times in all. The albumin solution was always brought to the same volume as that of the original diluted serum before the addition of the saturated ammonium sulphate solution.

The total globulin of the serum was dissolved and reprecipitated five times in the case of ox serum and six times in the case of horse serum. The globulin after each precipitation was dissolved in distilled water and the solution was diluted until the volume was the same as that of the original diluted serum. A portion of the total globulin was then set aside for analysis and the remainder was separated into euglobulin and pseudo-globulin by Haslam's method [1913]. Each globulin was dissolved and reprecipitated five times in the case of ox serum and six times in the case of horse serum globulin.

The different proteins prepared as described above were finally dissolved in distilled water and dialysed against running tap water until free from ammonium sulphate. The aqueous solutions of the different proteins were evaporated to dryness in a modified form of the apparatus described by Martin [1896], a dropping funnel, the exit tube of which was drawn out to a fine capillary, being substituted for the Berkefeldt filter. The bath containing the two drying bottles was maintained at a temperature of 40° and the larger bottle which acts as a receiver was immersed in ice. The dried proteins obtained in this way were reduced to a fine powder and analysed.

Euglobulin was also prepared by the method of Panum [1851]. The serum was diluted ten times with distilled water and normal acetic acid added in sufficient amount to precipitate the euglobulin. The amount of acetic acid necessary for complete precipitation was previously determined by tests on small quantities of the diluted serum. After standing overnight the clear supernatant fluid was decanted and the precipitated euglobulin centrifuged. It was purified by being dissolved in a minimum and measured quantity of decinormal sodium hydrate and reprecipitated by the addition of an exact equivalent of decinormal acetic acid. This process was repeated four times and the euglobulin was finally washed twice in the centrifuge tubes with distilled water.

A sample of the original serum was dried in Martin's apparatus.

Methods of Analysis.

In carrying out the analyses of the different serum proteins the experimental conditions were kept as constant as possible. From four to five grams of material were boiled with 80 cc. of 20 % hydrochloric acid and the rate of hydrolysis was followed by testing samples of the fluid at intervals in van Slyke's apparatus. The hydrolysis was continued for 20 to 22 hours.

After hydrolysis of the protein the excess of hydrochloric acid was removed *in vacuo* and the hydrolysed material washed into a 250 cc. flask. Duplicate quantities of 20 cc. of the solution were used for the determination of the total nitrogen, and 200 cc. of the solution were used in all cases for analysis. The sum of the ammonia nitrogen, melanin nitrogen, total nitrogen of the filtrate, and the total nitrogen of the bases (the last-named being determined in two parts) should be the same as the amount originally present, as determined separately in the 20 cc. portions. The experience gained in the study of these serum proteins shows that with the exercise of ordinary care throughout the course of the analysis it is not difficult to recover the whole of the nitrogen.

In separating the diamino acids from the monamino acids the same amount of phosphotungstic acid (15 g.) was used, and the precipitation was carried out at a volume of 200 cc. in all cases. After the addition of the phosphotungstic acid the mixture was heated on the water bath until most of the precipitate had redissolved, and after cooling to room temperature the vessel was allowed to stand for 48 hours in the cold room. The precipitate of the bases was washed with an ice-cold solution of phosphotungstic acid in hydrochloric acid, 120 to 140 cc. of solution being used.

TABLE III.

Details of observations.

Material	Quantity taken g.	Total N: cc. N/10 acid per 20 cc.	Total N: g. N per 200 cc.	Ammonia: cc. N/10 acid per 200 cc.	Ammonia: g. N per 200 cc.	Melanin: cc. N/10 acid per 200 cc.	Melanin: g. N per 200 cc.	Cystine: g. $BaSO_4$ per 40 cc.	Cystine: g. N per 200 cc.	Arginine: cc. N/10 acid per 100 cc.	Arginine: g. N per 200 cc.
A. Ox serum											
1. (*a*) Whole protein	4	24·2 24·1	0·3381	16·9	0·0237	3·8	0·0053	0·0240	0·0072	6·2	0·0347
(*b*) Whole protein	4	24·5 24·6	0·3437	17·6	0·0246	4·0	0·0056	0·0209	0·0063	6·1	0·0342
2. Albumin	4	27·9 28·0	0·3913	16·2	0·0277	3·3	0·0046	0·0380	0·0114	6·7	0·0375
3. Total globulin	4	26·5 26·4	0·3703	20·4	0·0286	5·5	0·0077	0·0170	0·0051	6·7	0·0375
4. Pseudo-globulin	4	27·2 27·2	0·3808	20·5	0·0287	5·2	0·0073	0·0160	0·0048	6·8	0·0381
5. Euglobulin $(NH_4)_2SO_4$ method	2·6	16·8 16·8	0·2352	15·6	0·0218	3·5	0·0049	0·0075	0·0022	4·3	0·0241
6. Euglobulin Panum's method	4	28·05 28·50	0·3958	22·7	0·0318	7·0	0·0098	0·0102	0·0031	7·7	0·0431
B. Horse serum											
7. (*a*) Whole protein	5·04	33·0 32·9	0·4613	23·7	0·0332	5·6	0·0078	0·0245	0·0073	7·3	0·0409
(*b*) Whole protein	4·95	33·0 33·2	0·4634	24·3	0·0340	5·0	0·0070	0·0241	0·0072	7·3	0·0409
8. (*a*) Albumin	4	29·5 29·5	0·4130	19·8	0·0277	3·1	0·0043	0·0341	0·0102	6·85	0·0384
(*b*) Albumin	4	29·4 29·7	0·4137	19·5	0·0273	2·9	0·0041	0·0345	0·0103	6·7	0·0375
9. (*a*) Total globulin	4	28·4 28·2	0·3962	22·6	0·0316	6·4	0·0090	0·0170	0·0051	5·2	0·0291
(*b*) Total globulin	4	29·2 29·4	0·4102	23·1	0·0323	6·9	0·0097	0·0115	0·0034	5·2	0·0291
10. Pseudo-globulin	5	35·7 35·6	0·4991	27·5	0·0385	7·8	0·0109	0·0203	0·0061	7·4	0·0414
11. (*a*) Euglobulin $(NH_4)_2SO_4$ method	4·59	32·3 32·3	0·4522	26·1	0·0365	7·3	0·0102	0·0176	0·0053	6·3	0·0353
(*b*) Euglobulin $(NH_4)_2SO_4$ method	4·62	32·8 32·9	0·4599	25·7	0·0360	7·6	0·0106	0·0162	0·0048	6·0	0·0336
12. Euglobulin Panum's method	4·5	29·5 29·5	0·4130	23·6	0·0330	6·9	0·0097	0·0159	0·0048	6·4	0·0358

TABLE III.

Details of observations.

Total N of Bases			Amino N of Bases		Amino N of Filtrate		Total N of Filtrate		
cc. N/10 acid per 100 cc.	cc. N/10 acid per 100 cc. + arginine titration	g. N per 200 cc.	cc. N per 40 cc.	g. N per 200 cc.	cc. N per 200/15 cc.	g. N per 200 cc.	cc. N/10 acid per 200/6 cc.	g. N per 200 cc.	Total N recovered g. per 200 cc.
					mm.				
29·0	35·2	0·0985	22·1; 16°; 768 mm.	0·0648	22·6, 22·7; 17°; 760	0·1962	24·6, 24·7	0·2071	0·3346
28·9	35·0	0·0980	22·5; 18°; 765 mm.	0·0651	22·7, 22·9; 16°; 768	0·2005	25·45, 25·50	0·2140	0·3422
41·4	48·1	0·1347	31·0; 16°; 774 mm.	0·0916	18·2, 18·4; 15°; 774[1]	0·2172	20·6, 20·7	0·2313	0·3933
27·4	34·1	0·0955	18·7; 17°; 763 mm.	0·0542	26·3; 19°; 762	0·2265	28·6, 28·4	0·2394	0·3712
26·7	33·5	0·0938	19·2; 18°; 764 mm.	0·0555	28·0, 27·8; 19°; 762	0·2400	29·9, 29·9	0·2512	0·3810
14·5	18·8	0·0526	10·6; 15°; 764 mm.	0·0310	16·1, 16·0, 16·2; 15°; 764	0·1415	18·3, 18·1	0·1529	0·2322
29·4	37·1	0·1039	19·5; 16°; 764 mm.	0·0568	20·0, 20·3; 16°; 764[1]	0·2349	22·0, 21·8	0·2453	0·3908
39·2	46·5	0·1302	29·5; 22°; 770 mm.	0·0843	32·4, 32·6; 20°; 773	0·2820	35·2, 35·2	0·2957	0·4669
38·5	45·8	0·1282	29·2; 21°; 765 mm.	0·0832	32·9, 33·0; 21°; 768	0·2829	35·5, 35·4	0·2978	0·4670
41·4	48·25	0·1351	31·6; 17°; 766·5 mm.	0·0184	27·3, 27·3; 17°; 766	0·2385	30·0, 29·9	0·2516	0·4188
40·5	47·2	0·1321	31·3; 17°; 766·5 mm.	0·0182	27·5, 27·6; 18°; 766	0·2394	29·7, 29·7	0·2495	0·4130
28·0	33·2	0·0930	20·8; 20°; 770 mm.	0·0600	28·8, 28·9; 19°; 773	0·2517	31·7, 31·7	0·2663	0·3999
27·5	32·7	0·0916	20·9; 20°; 764 mm.	0·0598	30·5, 30·4; 19°; 770	0·2640	32·9, 33·0	0·2768	0·4104
35·9	43·3	0·1212	25·8; 21°; 764 mm.	0·0734	36·7, 36·7; 22°; 763	0·3114	39·4, 39·4	0·3309	0·5015
30·8	37·1	0·1039	22·8; 18°; 752 mm.	0·0648	33·6, 33·4; 18°; 752	0·2856	35·8, 36·0	0·3015	0·4521
32·4	38·4	0·1075	23·4; 20°; 762 mm.	0·0667	34·2, 34·4; 18°; 752	0·2925	36·4, 36·5	0·3062	0·4603
29·1	35·5	0·0994	21·9; 24°; 757 mm.	0·0608	31·1, 31·1; 25°; 755	0·2572	32·0, 32·2	0·2696	0·4117

[1] Solution brought to 200 cc.

One of the more difficult of the estimations in the process is the determination of the total nitrogen of the bases, which is carried out on the same solution used for the determination of the arginine. In these analyses the practice was adopted of continuing the digestion for some time after the solution becomes clear, gently moving the fluid round the sides of the flask from time to time in order to wash down into the acid any particles which may have crept on to the upper parts of the digestion flask. The figures for the histidine nitrogen show the widest variation in duplicate analyses. This value is not determined directly but is calculated from the results of three other determinations, viz. the arginine nitrogen, the amino nitrogen of the bases, and the total nitrogen of the bases. As van Slyke points out these three values can all be determined accurately, and the duplicate analyses for the histidine should not vary by more than one per cent. of the total nitrogen.

The following estimations were carried out on the solution of hydrolysed protein:

(1) Total nitrogen was estimated in 20 cc. of the original solution.

(2) Ammonia nitrogen and melanin nitrogen were estimated in 200 cc. of the original solution.

(3) After removal of the ammonia and melanin the separation with phosphotungstic acid was carried out.

(4) The filtrate from the phosphotungstic acid precipitate of the bases was made to 150 cc. and the following estimations made:

(*a*) Total nitrogen of the filtrate in 25 cc.

(*b*) Amino nitrogen of the filtrate in 10 cc.

(5) The solution of the bases was made to 50 cc. and the following estimations made:

(*a*) Cystine nitrogen in 10 cc.

(*b*) Amino nitrogen in 10 cc.

(*c*) Arginine nitrogen and total nitrogen of the bases in the same sample of 25 cc.

The detailed figures obtained in the analysis of the different serum proteins are given in the tables. Table III (pp. 548, 549) contains a record of the actual observations, the volumes given in the headings referring in every case to the amount of the original solution corresponding to the volume actually employed as explained above. In Table IV the results are expressed as percentages of the total nitrogen.

TABLE IV.

Results of analyses expressed in percentages of total nitrogen.

Material	Ammonia N	Melanin N	Cystine N	Arginine N	Histidine N	Lysine N	Amino N of Filtrate	Non-Amino N of Filtrate	Total N recovered
A. Ox serum									
1. (*a*) Whole protein	7·0	1·6	2·1	10·2	3·4	13·3	58·0	3·2	98·8
Corrected for solubility of bases			2·9	11·2	4·5	13·4	56·5	1·8	
(*b*) Whole protein	7·1	1·6	1·8	9·9	3·2	13·5	58·3	3·9	99·3
Corrected			2·6	10·9	4·3	13·7	56·8	2·5	
2. Albumin	5·8	1·1	2·9	9·6	5·7	16·2	55·5	3·6	100·4
Corrected			3·5	10·4	6·7	16·3	54·2	2·3	
3. Total globulin	7·7	2·0	1·4	10·1	5·3	8·9	61·1	3·4	99·9
Corrected			2·0	10·9	6·3	9·0	59·8	2·2	
4. Pseudo-globulin	7·5	1·9	1·2	10·0	3·9	9·5	63·0	3·0	100·0
Corrected			1·9	10·8	4·8	9·6	61·7	1·6	
5. Euglobulin $(NH_4)_2SO_4$ method	9·3	2·0	0·9	10·2	2·2	9·0	60·1	4·9	98·6
Corrected			2·0	11·6	3·8	9·2	57·9	2·8	
6. Euglobulin Panum's method	8·0	2·5	0·8	10·9	5·6	9·0	59·3	2·6	98·7
Corrected			1·4	11·7	6·5	9·1	58·0	1·4	
B. Horse serum									
7. (*a*) Whole protein	7·2	1·7	1·6	8·8	4·9	12·8	61·1	3·0	101·1
Corrected			2·1	9·5	5·8	12·9	60·0	1·9	
(*b*) Whole protein	7·3	1·5	1·5	8·8	4·6	12·6	61·0	3·2	100·5
Corrected			2·1	9·5	5·4	12·7	59·9	2·1	
8. (*a*) Albumin	6·7	1·0	2·4	9·3	5·1	15·7	57·7	3·1	101·0
Corrected			3·1	10·0	6·1	16·0	56·5	2·0	
(*b*) Albumin	6·6	0·9	2·5	9·0	4·6	15·7	57·8	2·4	99·5
Corrected			3·1	9·8	5·6	15·8	56·6	1·2	
9. (*a*) Total globulin	8·0	2·3	1·3	7·3	4·1	10·6	63·5	3·7	100·8
Corrected			1·9	8·1	5·1	10·7	62·2	2·4	
(*b*) Total globulin	7·9	2·3	0·8	7·1	3·6	10·7	64·3	3·1	99·8
Corrected			1·4	7·9	4·5	10·9	63·1	1·9	
10. Pseudo-globulin	7·7	2·2	1·2	8·3	5·0	9·7	62·4	3·9	100·4
Corrected			1·7	8·9	5·8	9·8	61·3	2·9	
11. (*a*) Euglobulin $(NH_4)_2SO_4$ method	8·0	2·2	1·1	7·8	4·2	9·8	63·1	3·5	99·7
Corrected			1·7	8·5	5·0	9·9	62·0	2·4	
(*b*) Euglobulin $(NH_4)_2SO_4$ method	7·8	2·3	1·1	7·3	5·1	9·9	63·6	3·0	100·1
Corrected			1·6	8·0	5·9	10·0	62·4	1·9	
12. Euglobulin Panum's method	8·0	2·3	1·1	8·7	4·3	10·0	62·3	3·0	99·7
Corrected			1·8	9·4	5·2	10·1	61·0	1·8	

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LVII. THE GELATINISATION OF PECTIN IN SOLUTIONS OF THE ALKALIES AND THE ALKALINE EARTHS.

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Pectin substances have been known since the year 1825, when Bracannot[1] extracted from the roots and stems of a number of plants a substance to which he gave the name of pectic acid; about the same time Payen extracted a similar substance from the root of *Ailanthus glandulosa*. This discovery was followed up by other investigators, and Frémy's researches [1848] placed beyond doubt the existence in plant tissues of a class of substances distinct from the celluloses, though strongly resembling them. Frémy shewed that the parent substance, to which he gave the name of pectose, exists together with cellulose in the cell walls of green fruits, as well as in other parts of plants, and passes into solution during the process of ripening; it may also be brought into solution by boiling with acids. To this soluble variety he gave the name of pectin. Pectin undergoes further changes in boiling, and Frémy recognised two distinct varieties, parapectin, which is precipitated by neutral lead acetate, and metapectin, which is produced by boiling pectin or parapectin with acids, and is precipitated by barium chloride. Frémy also discovered the coagulating action of the enzyme pectase. These results have been confirmed and extended by later observers, especially by Mangin [1892, 1893], and a series of pectin substances has been recognised, differing in solubility, coagulable under different conditions, and marked by increasing acidity.

The action of alkalies on pectin was regarded by Frémy and his successors as resulting in the formation of an acid substance to which the name of pectic acid has been given; this being insoluble is precipitated by acids from alkaline solutions of pectin. The insoluble compounds of the alkaline earths

[1] For a résumé of early researches on pectin, see *Science Progress*, **6**, 344.

were looked upon as salts of pectic acid, and the action of pectase has been ascribed to the formation of calcium pectate. These conclusions of Frémy have been accepted by later observers; nevertheless the existence of pectic acid, and in particular its action in determining gelatinisation, must be regarded as in the highest degree uncertain. The work of recent years on adsorption compounds has taught us that change of physical properties and state of aggregation frequently occurs without any definite change of chemical composition. Gelatinisation and loss of solubility cannot be regarded as evidence in themselves of chemical change. All that is definitely known of pectin in this connection may be summarised as follows:

(1) Pectin boiled with water and acids becomes increasingly acid in reaction.

(2) It is coagulated reversibly by the alkalies, irreversibly by the alkaline earths; the action of acids on this coagulum leaves in both cases an insoluble residue.

(3) Certain salts, e.g. calcium chloride, in very concentrated solution, bring about a coagulation which is reversible.

(4) Coagulation by pectase is dependent upon the presence of calcium salts [Bertrand and Mallevre, 1894].

Although this last fact can be simply explained by the supposition that pectic acid is produced and is precipitated in the form of its calcium salt, this is quite uncertain, and the identification of this salt with the product of the reaction of pectin with calcium hydroxide is entirely unsupported by evidence.

The chemical composition of pectin has not at present been determined with any certainty. Analyses vary considerably but indicate a composition very near to, if not exactly, that of a carbohydrate; from the fact that pectin substances give furfural on distillation with hydrochloric acid and mucic acid when treated with nitric acid, the presence of galactan and pentosan groups in the complex molecule has been deduced. The analyses indicate that there may be a slight excess of oxygen in the molecule above the carbohydrate percentage, and Tollens [1914] has suggested that this may be explained by the existence of a few carboxyl groups in the molecule; if these are condensed with hydroxyl in the neutral pectin their progressive hydrolysis would account for the increasing acidity of pectin solutions when these are hydrolysed. In conformity with this hypothesis Tollens suggests a formula $6\,(C_{10}H_{18}O_9) + O$ or $5\,(C_{10}H_{18}O_9) + C_{10}H_{18}O_{10}$.

The object of the experiments described below was the study of the mechanism of gelatinisation and its dependence upon chemical change in the case of pectin solutions gelatinised by the action of the alkalies and alkaline earths.

Experimental.

The pectin used in the following experiments was prepared principally from limes; a little lemon pectin was also used. The fruit was cut up and boiled with water for several hours until quite soft; the liquid was then filtered through muslin and the residue further extracted with boiling water, until the greater part of the pectin had been removed. The liquid was evaporated to a small volume, alcohol was added to precipitate the pectin, and the jelly thus produced was drained through muslin, redissolved in water, and reprecipitated by alcohol many times. No attempt was made to remove calcium salts. The solutions were finally boiled until all alcohol was driven off. With the specimens prepared later solutions were made from the dry powder obtained by washing the pectin jelly with alcohol until most of the water was removed, drying at 100° and powdering. The solutions were distinctly acid and highly viscous, they could be kept indefinitely in the presence of chloroform; a slight sediment was formed on standing apparently due to the presence of mechanically contained impurities. Pectin prepared in this way is readily precipitated by neutral lead acetate, and therefore consists largely, if not entirely, of the variety distinguished by Frémy and Mangin as parapectin; barium chloride gives no precipitate but concentrated solutions form a jelly on standing.

The following are particulars of the different pectin solutions used:

(*a*) Prepared from limes; concentration (determined by evaporating 5 cc. at 100°) 0·48 g. in 100 cc.

(*b*) Prepared from limes; concentration 0·25 g. (dry powder) in 100 cc.; ash 2·72 per cent.

Acidity 10 cc. = 0·5 cc. N/25.

(*c*) Prepared from lemons; concentration approx. 0·25 g. (dry powder in 100 cc.); determined by evaporating 5 cc. at 100° = 0·31 g. in 100 cc.

Acidity 10 cc. = 0·3 cc. N/25.

The weight of residue left by evaporating solution (*b*) to dryness was not determined; the concentrations of (*b*) and (*c*) were nearly the same, (*b*) being slightly the more concentrated.

Measurements of the Rate of Precipitation of Pectin by the Alkalies and Alkaline Earths.

If solutions of the alkaline earths are added to a solution of pectin containing 1 per cent. or even less, a stiff jelly is produced; concentrated solutions of potassium hydroxide produce a similar effect. If the pectin solution is very dilute, a gelatinous precipitate is formed, resembling in appearance a small precipitate of aluminium hydroxide; if the alkali is also sufficiently dilute, the rate of precipitation becomes measurable. In very dilute solutions, if the mixture is allowed to stand without shaking, a very attenuated jelly is formed; this is very unstable; a slight movement causes rifts to form in the jelly, and precipitation takes place almost instantaneously. From this it appears that the first action of barium hydroxide or other alkali is the formation of a continuous jelly, which being mechanically unstable is readily broken up in dilute solution; with a larger excess of hydroxide the mechanical instability is so great that precipitation takes place immediately. That a jelly is formed in the first place is confirmed by the more or less sudden change which takes place in the appearance of the mixture in all cases previous to precipitation. With potassium hydroxide the action is very similar, but very much higher concentrations are necessary for gelatinisation and precipitation, and the precipitate forms less sharply; the rate of precipitation is consequently less easily measured.

The following method was employed for the measurement of the rates of precipitation: the solution of alkali or of alkaline earth was measured into a small flask and diluted to a volume such that with the addition of pectin solution it measured 50 cc. During the experiment the temperature was kept constant (usually at 15°) by immersing the flask in a glass trough filled with water. To ensure immediate precipitation the flask was constantly shaken. The time was measured from the moment at which the addition of pectin solution was made to the moment of precipitation; it was found possible to measure to within a quarter of a minute, and the results obtained usually agreed to within half a minute or less. The pectin solution used for all rate measurements was the solution (*a*) unless otherwise mentioned.

Precipitation by the hydroxides of the alkaline earth metals.

It was found that in all cases the rate of precipitation was nearly proportional to the square of the concentration; in the following tables the product time $\times$ (concentration)^2 is given for comparison.

In the diagram (Fig. 1) the reciprocals of the times are plotted against the squares of the concentrations.

The effect of change of temperature has not been measured, but the rate increases very rapidly with a small increase of temperature.

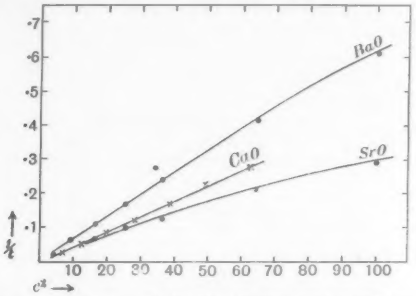

Fig. 1. Rate of precipitation of pectin by the alkaline earths (Tables II B, III and IV).

TABLE I.

Rate of precipitation of pectin by barium hydroxide.

Conc. of BaO_2H_2 mg. equiv. per litre	A. 2 cc pectin in 50 cc.		B. 1 cc. pectin in 50 cc.		C. 0·5 cc. pectin in 50 cc.	
	t (mins.)	c^2t	t (mins.)	c^2t	t (mins.)	c^2t
c	1 measurement		1 measurement		2 measurements	
2	35	140	30	120	34	136
3	—	—	—	—	16[1]	144
4	8·5	136	9	144	9·25	148
6	4	144	4	144	5	180
8	2·5	160	3	192	3	192
10	1·5	150	2	200	2	200

[1] 1 measurement.

Another series gave the following results :

TABLE II.

Rate of precipitation of pectin by barium hydroxide.

Conc. of BaO_2H_2 mg. equiv. per litre	A. 3 cc. pectin in 50 cc.		B. 2 cc. pectin in 50 cc.	
	t (mins.)	c^2t	t (mins.)	c^2t
c	1 measurement		2 measurements	
2	47·5	190	41·9	167
3	17[1]	153	16·25	146
4	9·25	148	9·1	146
5	—	—	6·0	150
6	4	144	4·1	148
8	2·5	160	2·4	152
10	1·75	175	1·6	162

[1] 2 measurements.

The series in Table II is the more accurate; in the most dilute pectin solutions the precipitate does not form very sharply.

TABLE III.

Rate of precipitation of pectin by strontium hydroxide.

Conc. of SrO_2H_2 mg. equiv. per litre c	2 cc. pectin in 50 cc. t (mins.) 2 measurements	c^2t
4	17·25	276
5	10·0	250
6	7·6	274
8	4·6	296
10	3·4	337

TABLE IV.

Rate of precipitation of pectin by calcium hydroxide.

Conc. of CaO_2H_2 mg. equiv. per litre c	A. 2 cc. pectin in 50 cc. t (mins.) 2 measurements	c^2t	B. 1 cc. pectin in 50 cc. t (mins.) 2 measurements	c^2t
2·6	37·4	253	30·9	209
3·5	19·25	236	16·9	206
4·4	11·5	223	11·0	213
5·3	8·1	228	7·9	222
6·2	6·0	231	6·5	250
7·0	4·5	220	4·75	233
7·9	3·6	226	3·9	242

A direct comparison was made of the rates of precipitation by barium, strontium, and calcium hydroxides in equivalent quantities. The following results were obtained:

TABLE V.

mg. equiv. per litre c	3 cc. of pectin solution (c) in 50 cc. t (mins.) Ba		Sr		Ca	
4	14		18		17	
3·8	6·5		9	9·75	7·5	8
9·9	2·5	2·5	3·75	4	3·25	3·25

In comparing the foregoing results, it must be remembered that the time measured is that necessary for precipitation, and not that which is required for the formation of the jelly. As soon as the jelly forms absorption of hydroxide takes place, and this causes the framework of the jelly to stiffen and contract, so that spontaneous precipitation takes place at all but very

small concentrations; at these concentrations precipitation can be brought about by shaking, but it is probably necessary in this case also for a certain amount of absorption to take place in the jelly before it can be precipitated. This amount will obviously vary with the character of the jelly, and is probably less for barium, which gelatinises very readily, than for strontium or calcium. The rate of absorption will depend upon the concentration of the hydroxide and the absorption capacity of the jelly; the latter factor is markedly greater for calcium than for the other hydroxides.

The abnormality of the strontium curve in position and character is probably due to the influence of the absorption factor in precipitation, which

TABLE VI.

Rate of precipitation of pectin solutions of varying concentrations by barium hydroxide (conc. 4 mg. equiv. per litre).

Conc. of pectin cc. solution (*a*) in 50 cc.	*t* (mins.) I.			II.
1	16·5	16·5	14	15·5
2	13	13·5		—
3		10·5		10·75
4		10		—
5		9·5		9·5
10		9·5		8·5
15		9		—
20		9		—
25		9		—
30		—		9·5
40		10		—
50		—		10·5

becomes unimportant for barium on account of the insolubility of the jelly, and for calcium on account of the rapidity with which absorption takes place. The effect will be that a quantity will be added to the time required for gelatinisation, which will decrease as the concentration increases, but will increase relatively to the total time of precipitation, thus causing the precipitation constant to increase. A slight increase of the precipitation constant is to be observed in the barium curves also. This increase in the precipitation constant cannot be ascribed to changes in the degree of dissociation, even where, as for barium hydroxide, the change would be of a corresponding order of magnitude in the two cases, for the conductivity curve shews that at the concentrations employed barium hydroxide dissociates according to the equation

$$BaOH^{\cdot} = Ba^{\cdot\cdot} + OH',$$

no non-ionised hydroxide being present, and it will be evident from the results and conclusions to be given below that the formation of $BaOH^{\cdot}$ ions is unlikely to affect the rate of precipitation. These remarks anticipate results and conclusions which will be given in detail at a later stage.

The effect of varying the quantity of pectin is shewn in Tables VI and VII.

TABLE VII.

Rate of precipitation of pectin solutions of varying concentrations by strontium hydroxide (conc. 5 mg. equiv. per litre).

Conc. of pectin cc. solution (*a*) in 50 cc.	*t* (mins.)
1	10·25
3	10·25
4	10·50
5	11·75

These tables shew that for very dilute solutions the rate of precipitation increases with increase in the quantity of pectin present; for larger concentrations an opposite effect is produced and the rate of precipitation gradually decreases.

The effect of salts on precipitation.

Potassium chloride added to a solution of barium hydroxide retards the precipitation of pectin; this is to be expected as double decomposition will take place to some extent.

TABLE VIII.

Rate of precipitation of pectin by barium hydroxide in the presence of potassium chloride.

2 cc. of pectin solution in 50 cc.
Conc. of BaO_2H_2 4 mg. equiv. per litre.

Conc. of KCl mg. mols. per litre	*t* (mins.)
0	9·1
10	10·75
20	11·5

The effect of salts having a common ion is to increase the rate of precipitation; it has been possible to measure the accelerating effects for both ions, since potassium hydroxide in dilute solution has itself no precipitating action on pectin.

TABLE IX.

Rate of precipitation of pectin by barium hydroxide in the presence of salts having a common ion.

A. 2 cc. of pectin solution in 50 cc.
Conc. of BaO_2H_2 4 mg. equiv. per litre.

	t (mins.)		
	$BaCl_2$		KOH
Conc. mg. equiv. per litre	I. (1 measurement)	II. (2 measurements)	(1 measurement)
2	7	7·25	6·25
4	6	5·75	4·75
6	5	4·75	3·5
8	4	4·25	3·25
10	3·5	3·75	3·00

B. 2 cc. of pectin solution in 50 cc.
Conc. of BaO_2H_2 2 mg. equiv. per litre.

	t (mins.)	
Conc. mg. equiv. per litre	$BaCl_2$ (2 measurements)	KOH (1 measurement)
2	21·4	17·5
4	16·4	11·75
6	13·4	10
8	11·75	8
10	9·5	—
12	9·25	—
20	—	3·25

C. 0·5 cc. of pectin solution in 50 cc.
Conc. of BaO_2H_2 4 mg. mols. per litre.

	t (mins.)	
Conc. mg. equiv. per litre	$BaCl_2$ (1 measurement)	KOH (1 measurement)
2	8·5	7
4	7	5·5
6	6·5	5
8	5·5	4·5
10	5·5	3·5
20	4·5	—

The corresponding measurements for calcium chloride were found to be partially invalidated by impurities in the calcium chloride and are therefore omitted; the results were however similar. Tables of the effect of strontium chloride and nitrate on strontium hydroxide and of potassium hydroxide on calcium hydroxide are given.

TABLE X.

Rate of precipitation of pectin by strontium hydroxide in the presence of strontium salts.

2 cc. of pectin solution in 50 cc.
Conc. of SrO_2H_2 4 mg. equiv. per litre.

Conc. (mg. equiv. per litre) $Sr(NO_3)_2$ and $SrCl_2$	t (mins.)	
	$Sr(NO_3)_2$ (3 measurements)	$SrCl_2$ (1 measurement)
2	10·75	12
4	8·6	10
6	7·6	9·25
8	6·9[1]	—
10	6·25	7·75
14	5·25	6·25
20	4·65	5·25

[1] 2 measurements.

TABLE XI.

Rate of precipitation of pectin by calcium hydroxide in the presence of potassium hydroxide.

2 cc. of pectin solution in 50 cc.
Conc. of CaO_2H_2 3·5 mg. equiv. per litre.

Conc. of KOH (mg. equiv. per litre)	t (mins.) (2 measurements)
2	13·1
4	9·9
6	8·75
8	7·1
10	6.4
12	6·0
20	4·5

It will be observed from these tables that the rate of precipitation is accelerated both by potassium hydroxide and by the salts of the alkaline earth metals; the effect of the latter is less than that of potassium hydroxide. It is of course possible that the anion exerts an inhibitory effect; but this is an unlikely supposition since, as is shewn later, the effect of potassium salts on the rate of precipitation by potassium hydroxide is nearly identical, and since chloride ions are not appreciably absorbed by the pectin jelly. The absorption of other anions has not been determined, but there is no reason to suppose the chloride ion in any way exceptional. A large part of the difference in the rates for barium chloride and potassium hydroxide is certainly due to the incomplete dissociation of the former; the uncertainty

as to the dissociation of divalent salts makes it difficult to say whether the whole difference can thus be accounted for, but the similarity of the two curves (see Fig. 2) makes this very probable; it is possible however that a small part of the discrepancy may be due to absorption effects. The conductivity curves for strontium chloride and nitrate are very uncertain at great dilutions; they however indicate that the dissociation of strontium chloride falls off with considerably greater rapidity than that of the nitrate as concentration increases, and this difference probably suffices to account for the small differences in the rates measured.

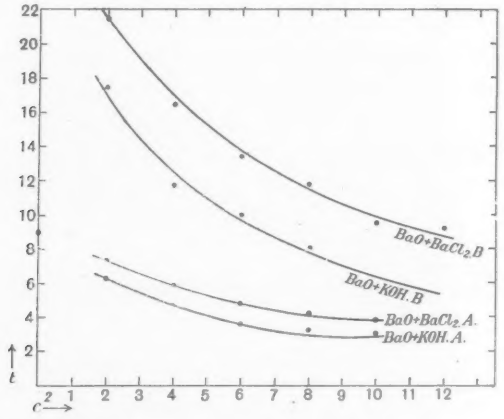

Fig. 2. Rate of precipitation of pectin by barium hydroxide in the presence of barium chloride and potassium hydroxide (Table IX A, B).

Since the rate of gelatinisation of pectin is proportional to the square of the concentration of the hydroxide added, and is accelerated by the addition of either ion, it is concluded that the rate of gelatinisation is proportional to the product of the concentrations of alkaline earth metal and hydroxyl; the following table gives the value of this product for equal times in the case of:

(1) barium hydroxide (C_1),

(2) barium hydroxide and barium chloride (C_2),

(3) barium hydroxide and potassium hydroxide (C_3),

assuming complete dissociation; the values of the concentrations for equal times are obtained by interpolation from the results in Table II B and Table IX A.

TABLE XII.

Value of the product $Ba \times OH$ for equal values of t.

t	BaO_2H_2 C_1[2]	$BaCl_2$ + 4 mg. equiv. BaO_2H_2 per litre $4(C_2+4)$	KOH + 4 mg. equiv. BaO_2H_2 per litre $4(C_3+4)$
3	52	—	56
4	36	46	35
5	29	37	30
6	23	30	20
7	18	21	—

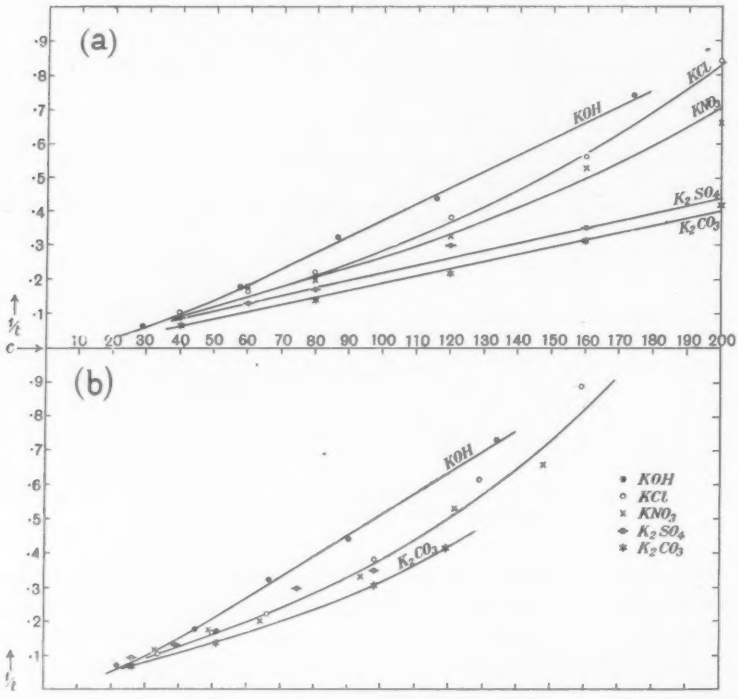

Fig. 3. Rate of precipitation of pectin by potassium hydroxide and by mixtures of potassium hydroxide and potassium salts.

(*a*) c = conc. of potassium salts in the presence of 290 mg. equiv. per litre of potassium hydroxide.

(*b*) c = conc. of potassium ions above 244 mg. equiv. per litre.

As will be seen the values for additions of potassium hydroxide correspond very closely with those for barium hydroxide alone; for barium chloride the percentage difference decreases as the concentration decreases.

Precipitation by Potassium Hydroxide.

TABLE XIII.

Rate of precipitation of pectin by potassium hydroxide.

2 cc. of pectin solution in 50 cc.

Conc. (c) mg. equiv. per litre	t (mins.) 2 measurements	$(C - 310)\,t$
319	15·15	186
348	5·75	218
377	3·1	209
406	2·25	216
464	1·4	212

The values of $1/t$ plotted against the concentration (Fig. 3 (a)) give a straight line cutting the x axis at the concentration 310; the values of the constant $(C - 310)\,t$ are given in the table.

The following table shews the effect on the rate of precipitation of additions of various potassium salts to potassium hydroxide.

TABLE XIV.

Rate of precipitation of pectin by potassium hydroxide in the presence of potassium salts.

2 cc. of pectin solution in 50 cc.
Conc. of KOH 290 mg. equiv. per litre.

Conc. mg. equiv. per litre	KCl t (mins.) 2 measurements	KNO_3 t (mins.) 2 measurements	K_2CO_3 t (mins.) 2 measurements	K_2SO_4 t (mins.) 2 measurements
40	9·5	9·0[1]	14	10·75
60	6·0	5·75	—	7·50
80	4·5	5·0	7·25	6·0
120	2·6	3·0	4·50	3·4
160	1·6	1·9	3·25	2·9
200	1·1	1·75	2·4	2·4

[1] 1 measurement.

Fig. 3 (a) shews these results graphically, the reciprocals of the values of t being plotted against the concentrations; the curves thus obtained only diverge slightly from a straight line, except in the case of potassium chloride.

If however the values of $\frac{1}{t}$ are plotted against the concentrations of the potassium ion, a different result is obtained; the curves for KCl, KNO_3 and K_2SO_4 fall together at the lower concentrations, though they diverge slightly as the concentration increases, and the curves for KCl and KNO_3 for which

the measurements extend further than those for the salts of the dibasic acids shew marked curvatures towards the y axis. For the sake of clearness the mean curve for the three salts is given in the figure (Fig. 3 (b)); the curve for potassium carbonate is shewn separately. The discussion of these results is deferred until later.

The calculation of the ionic concentrations was made on the assumption that for salts of monobasic acids $C_3 = C_2(C_1 + C_2)$ and of dibasic acids $C_3 = C_2(C_1 + C_2)^2$, where

C_1 = concentration of KOH,

C_2 = concentration of salt,

C_3 = that concentration at which the salt in pure solution is equally dissociated.

If the square root or cube root of the product of the ions be taken to represent the effective ionic concentration, we have

$$C_3^2 \alpha_3^2 = C_2(C_1 + C_2)\gamma_1 \alpha_2$$

and

$$C_3^3 \alpha_3^3 = C_2(C_1 + C_2)^2 \gamma_1^2 \alpha_2,$$

where α_1 represents the mean dissociation of the mixture of salt and potassium hydroxide; for salts of monobasic acids $\alpha_1\alpha_2 = \alpha_3^2$ approximately; the formula will therefore enable the degree of dissociation of the salt in the mixture to be calculated with fair accuracy; for salts of dibasic acids $\alpha_1^2\alpha_2 > \alpha_3^3$, the value found for C_3 will therefore be too small and the degree of dissociation too high; for small concentrations however the percentage error is not very great.

The following table gives the data for the calculation of the degree of dissociation of the various salts between the concentrations 40 and 200 mg. equiv. per litre in the presence of potassium hydroxide at a concentration of 290 mg. equiv. per litre.

TABLE XV.

Salt	Concentration (c)	C_3	λ	λ_∞	α
KCl	40	115	1040	1240	84
	200	313	985	—	79·5
KNO_3	40	115	970	1180	82
	200	313	875	—	74
K_2CO_3	40	163	850	1300	65·5
	200	363	775	—	59·5
K_2SO_4	40	163	855	1310	65·5
	200	363	780	—	59·5
KOH	290	290	1905	2270	84
	319	319	1895	—	83·5
	464	464	1853	—	81·5

The concentrations of dissociated salt (C) corresponding to the concentrations in Tables XIII and XIV and the product of these concentrations with α are therefore as follows:

TABLE XVI.

A. KOH.

C	αc
290	244
319	266
348	289
377	311
406	334
464	378

B. *Salts.*

	KCl	KNO_3	K_2CO_3	K_2SO_4
C	αC	αC	αC	αC
40	34	33	26	26
60	50	49	39	39
80	66	64	51	51
120	98	94	75	75
160	129	122	98	98
200	159	148	119	119

The decrease in the dissociation of KOH with increasing salt concentrations has been disregarded.

The following table shews the action of potassium chloride on varying quantities of potassium hydroxide:

TABLE XVII.

5 cc. of pectin solution (c) (from lemons) at 20°.

Conc. mg. equiv. KOH per litre	Conc. mg. equiv. KCl per litre	t (mins.) 1 measurement
686	0	1·5
343	200	2·5
171	300	5·25
103	340	14

Absorption of the Alkaline Earths by Gelatinised Pectin.

The process of gelatinisation is accompanied by absorption; in dilute solution the precipitate can be easily filtered off, and it was possible therefore to determine by titration the quantity of hydroxide that had been absorbed. Since the change of volume on gelatinisation is inappreciable, the following method was adopted for the estimation of the amount of hydroxide absorbed.

A mixture of alkaline earth, pectin and water was made in a flask of known volume and was allowed to stand for a definite time, usually half an hour.

It was then filtered, the first part of the filtrate was rejected and a part of the remainder, usually one-half of the original volume, was titrated with standard acid.

To avoid absorption of carbon dioxide the filtering funnel was fixed in a bell jar, through which passed a funnel provided with a stopcock; a little potash was placed inside the bell jar, and all communications to the outer air were closed with soda lime tubes. After standing, the contents of the flask were emptied rapidly into the stoppered funnel; the first part of the filtrate was then allowed to run through, after which the whole apparatus was removed, so that the filter drained into a dry flask. To ensure constant temperature the whole apparatus, together with the water and solutions, used for the mixture, was kept in a zinc cupboard, maintained at a constant temperature by means of a thermoregulator. A variation of 5° did not affect the results appreciably.

Absorption takes place rapidly, and, except in the case of the most dilute solutions, apparent equilibrium is established in less than half an hour; the following measurements shew the variation in the rate of absorption in very dilute solution.

TABLE XVIII.

Rate of absorption of barium hydroxide in dilute solution by gelatinised pectin.

Volume 100 cc. 10 cc. of pectin solution (*C*).

Final conc. of BaO_2H_2 100 cc. = cc. N/25	Absorption		
	0·5 hr.	1 hr.	2 hrs.
5	1·7	2·0	2·3
	1·8	2·1	2·2
	1·7	—	—
11	2·1	2·2	2·3
	2·1	—	—
38	2·8	—	2·6
	2·6	—	—

It will be noticed that, except at extreme dilution, equilibrium was established within the half hour; the variations for the concentrations 11, though these probably indicate a slight increase, are within the errors of experiment. This however was not true equilibrium, as a slow absorption continued to take place even after several days; this slow absorption may be due to chemical action, but is more probably caused by slow diffusion of hydroxide into the interior of the jelly. A similar effect was observed by Morawitz [1910] in his experiments on the absorption of salts by charcoal,

and was ascribed by him to diffusion. In the case of calcium, which is much more strongly absorbed than either barium or strontium, the increased absorption is appreciable after 4·5–5 hrs. The following measurements for calcium and strontium are given for comparison.

TABLE XIX.

Absorption after 4·5–5 hrs.

Vol. 100 cc. 10 cc. of pectin solution (*C*).

A. *Strontium Hydroxide.*

Final conc. 100 cc. = cc. N/25	Absorbed after 0·5 hr.	Absorbed after nearly 5 hrs.
11	2·1	2·2
36	2·9	2·9
90	3·6	3·8

B. *Calcium Hydroxide.*

	after 0·5 hr.	after 4·5 hrs.
8	2·5	2·7
	2·5	—
	2·5	—
27–29	3·1	3·5
	3·2	after 24 hrs.
		4·8

In these experiments the absorption was determined by titrating with N/25 HCl and phenolphthalein in the cold; the alkaline earth solution was added from a pipette, which was washed out into the flask; water and pectin were then added, and the flask was filled to the mark and thoroughly shaken. In all the absorption experiments the water used was boiled to free it from carbon dioxide. The order in which the mixture was made—whether it was diluted before or after the addition of pectin—was found to be without effect. The concentration of hydroxide was determined for each measurement by a blank titration in which pectin was absent. The mixture was allowed to stand half an hour, and the amount of absorption was then measured by the difference in the value given by the titration of 50 cc. in the two cases, the mixture having been filtered as described above. The absorption at this point was assumed to have attained a definite state, probably by saturation of the outer layer of the jelly-forming substance; that this assumption is justified is confirmed by the regularity of the absorption curves obtained. Differences between two titrations giving the amount absorbed usually agreed within less than 0·1 cc.; the greatest variations amounted to 0·15. These are doubled in the table which expresses the absorption for the total volume.

TABLE XX.

Absorption of the alkaline earths by gelatinised pectin.

Vol. 100 cc. 10 cc. pectin solution (*C*). *T* 20°–25°.

BaO_2H_2 2 measurements		SrO_2H_2 1 measurement		CaO_2H_2 2 measurements	
Final conc. 100 cc. = cc. N/25	Absorbed cc. N/25	Final conc. 100 cc. = cc. N/25	Absorbed cc. N/25	Final conc. 100 cc. = cc. N/25	Absorbed cc. N/25
11	2·1	11	2·1	8	2·5
38	2·7	36	2·9	30	3·15
66	3·0	—	—	51	4·05
94	3·2	90	3·6	73	4·55

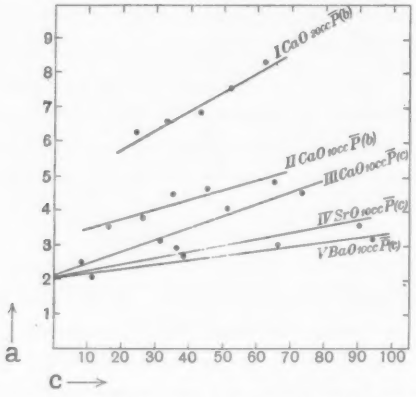

Fig. 4. Absorption of alkaline earths by pectin (Tables XX. XXI).

It will be seen (Fig. 4) that these points lie on converging straight lines which nearly meet on the *y* axis; if Table XVIII be referred to it will also be noticed that, even at very great dilutions, the amount of barium hydroxide absorbed coincides with the corresponding line, if sufficient time be allowed for equilibrium to be established. Strontium and calcium do not gelatinise pectin so readily, and in consequence absorption cannot be determined at equally great dilutions; this is especially the case for strontium.

It was unfortunately impossible to extend these results as material was lost during an interruption of the work, and the concluding experiments were made with a very limited quantity of pectin; some earlier measurements are however given, made with a different specimen of pectin, which shew the effect on the absorption of increasing the concentration of the pectin. The measurements are not quite so accurate as the preceding, the method

employed being slightly different; the concentration of the calcium hydroxide was in this case determined by titration of the original solution, and a measured volume of this solution was added from a pipette. This method had two defects, which were avoided by the method described above; the amount of carbon dioxide absorbed during the mixing process was unchecked, and the delivery of calcium hydroxide solution from the pipettes was not quite regular, this being due to a tendency of the solution to behave towards glass as to a greasy surface, in consequence of which drops were liable to adhere to the pipette. The flasks and pipettes used were carefully calibrated. In a given series the greatest differences between two measurements of the absorption was 0·3 c.c.

TABLE XXI.

Absorption of calcium hydroxide by gelatinised pectin.

Vol. 100 cc. T 18°–20°.

A. 20 cc. of pectin solution (*b*).

Final conc. 100 cc. = cc. N/25	Absorbed cc. N/25 Series I (2 measurements)	Series II (2 measurements)	Mean
24	6·3	6·15	6·25
33	(7·3)	6·55	6·55
43	6·9	6·8	6·85
52	7·45	7·65	7·55
62	7·65	7·95	7·8

B. 10 cc. of pectin solution (*b*).

Final conc. 100 cc. = cc. N/25	Absorbed cc. N/25 (2 measurements)
16	3·55
26	3·8
35	4·5
45	4·65
65	4·85

The mixture was left for half an hour as in the preceding measurements, and equilibrium was tested by measuring the absorption after 1 hour at low and high concentrations. The corresponding curves are shewn in Fig. 4 I, II.

Absorption in the presence of calcium or barium chloride.

A fairly concentrated solution of calcium chloride was necessary to increase the absorption by a measurable amount; the following table shews the increase in absorption for different concentrations of calcium hydroxide; 30 cc. or 70 cc. of approximately N/25 calcium hydroxide were mixed with 20 cc. of pectin and varying quantities of calcium chloride, and the volume was made up to 200 cc.

TABLE XXII.

Absorption of calcium hydroxide by gelatinised pectin in the presence of calcium chloride.

Vol. 200 cc. 20 cc. of pectin solution (*b*). *T* 18°–20°.

A. 70 cc. CaO_2H_2 added: final conc. 200 cc. = 62–66 cc. N/25.

cc. N/25 $CaCl_2$	Absorption (1)	(2)	(3)	Mean
0	7·25	7·25	7·25[1]	7·25
10	7·7	7·7	7·45	7·62
50	8·4	8·25	8·35	8·33

B. 30 cc. CaO_2H_2 added: final conc. 200 cc. = 23–26 cc. N/25.

0	5·8	5·8	5·85[1]	5·82
10	(6·9)	6·15	—	6·15
50	6·7	6·7	—	6·7

[1] After standing 1 hour.

In order to determine whether or not chloride ions were absorbed, 10 cc. of the filtrate from the mixtures A and B of the preceding table, each containing 50 cc. $CaCl_2$ in 200 cc., were neutralised and titrated with N/10 $AgNO_3$, potassium bichromate being used as indicator; 5 cc. of the original $CaCl_2$ solution required 23·95 $AgNO_3$. The equivalent quantities of the solutions to which pectin had been added required:

A. 23·8 cc.

B. 24 cc.

It appears therefore that pectin does not absorb chloride ions.

TABLE XXIII.

Absorption of barium hydroxide by gelatinised pectin in the presence of barium chloride.

Vol. 100 cc. 20 cc. of pectin solution (*b*). *T* 17°–18°.

A. Final conc. of BaO_2H_2: 100 cc. = 67–69 cc. N/25.

cc. N/2 $BaCl_2$	Absorbed (1)	(2)	Mean
0	6·6	6·5	6·55
10	8·2	7·9	8·05
30	8·0	—	8·0

B. Final conc. of BaO_2H_2: 100 cc. = 9–10 cc. N/25.

5	5·0	4·7	4·85
40	5·1	4·8	4·95

Table XXIII shews the absorption of barium hydroxide in the

presence of varying quantities of barium chloride; the concentration was determined for each measurement by a blank test in the absence of pectin; titrations were made by adding 20 cc. of N/2 HCl to the mixture where necessary, and titrating with N/25 HCl in the presence of phenolphthalein. In this case the solutions were boiled to eliminate carbon dioxide, but this was found to be without effect upon the result.

Theoretical.

It has been shewn that the rate of precipitation of pectin by the alkaline earths is very nearly proportional to the product of the concentrations of hydroxyl and metallic ion, and that this product may, with great probability, be taken as a measure of the rate of gelatinisation; the theoretical implications of these results remain to be considered.

(1) The fact that both anions and kations are concerned in the precipitation excludes the possibility that the alkali acts by a process of diffusion into the suspended pectin complex, since in this case the rate of diffusion and consequently of precipitation also, would be dependent upon the rate of diffusion of the more slowly moving ion; i.e. the rate of precipitation would be determined in general by the metallic ion alone.

(2) It is also improbable that precipitation is due to surface adsorption. This usually takes place with great rapidity and it is quite unlikely that the process of adsorption would be prolonged through a considerable interval, as in this case; moreover when adsorption occasions precipitation this has been found to be generally due to the adsorption of an ion having a charge opposite to that of the adsorbent [see especially Morawitz, 1910]. Since both positive and negative ions are concerned in the precipitation of pectin, this cannot be due to any change of potential brought about by adsorption.

(3) A third possibility is that the rate of gelatinisation is a measure of the rate of chemical change.

It may be seen from the measurements of absorption that the amount of alkaline earth which combines with pectin is small, therefore the concentrations of alkaline earth may be taken as constant throughout the time t, and we have on this assumption

$$\frac{dx}{dt} = RC_1C_2(a-x)^p,$$

where a is the initial quantity of pectin present; x is the amount transformed in time t; C_1, C_2 the concentrations of metallic ion and hydroxyl.

Putting $p = 1$ we have

$$RC_1C_2 = \frac{1}{t} \log \frac{a}{a-x}.$$

Now $\frac{a}{a-x}$ is constant for all the measurements of a series since it must be presumed that gelatinisation occurs when a definite quantity of pectin has undergone change.

Hence $$C_1C_2t = K.$$

It is therefore possible to express the rate of gelatinisation of pectin as determined by the velocity of a chemical change in which pectin reacts with barium, strontium or calcium and hydroxyl ions; the reaction equation will probably be of the form

$$\overline{P}. \cdot OH + BaO_2H_2 = \overline{P}. \cdot OBaOH + H_2O.$$

It may be observed in Tables I–IV that the value of the constant c^2t is usually too high for the first measurement of the series, and that the error is greater, the greater the quantity of pectin present; this may be accounted for not only by the slight acidity of the pectin but also by the fact that when the value of c is very small, an appreciable error is made in taking c as constant throughout the time t.

It has been shewn that the rate of precipitation of pectin by potassium hydroxide is conditioned by the concentration of potassium ions; it is to be remarked, however, that gelatinisation does not take place below a definite and fairly high concentration. Since above this concentration the rate of gelatinisation increases proportionately to the increase of concentration, it may be assumed that hydrolysis takes place, and that the reaction may be represented by the equation

$$\overline{P}. \cdot OH + KOH \rightleftarrows \overline{P}. \cdot OK + H_2O$$

or

$$\overline{P}. \cdot OH + K^{\cdot} + OH' \rightleftarrows \overline{P}. \cdot OK + H_2O$$

which takes place in both senses; the pectin cannot be itself dissociated since the reaction takes place slowly, and therefore could only take place between ions if it were dependent upon a process of diffusion, which has been shewn to be extremely improbable. The equation shews that the rate of reaction for a given concentration of hydroxyl will be determined by the concentrations of the potassium ions.

The hypothesis of chemical change affords therefore a partial explanation of the character of the curves shewn in Fig. 3. These curves indicate, however, that while for small additions of salt the rate of precipitation is nearly pro-

portional to the increase of the potassium ions, for larger additions the rate of precipitation increases more rapidly, the effect being ultimately greater than that produced by an equal quantity of potassium hydroxide; moreover the specific character of the salt begins to assert itself. This appears to point to a molecular reaction between salt and pectin, and this reaction as is to be expected is more evident in the case of the salts of monobasic than in that of those of dibasic acids. The curve for potassium carbonate suggests that the error in the calculation of the degree of dissociation is preceptibly greater in this case than in that of potassium sulphate, which is probable since the ordinary solution of potassium carbonate contains hydrolysed salt, whereas in the present case hydrolysis is prevented by the excess of potassium hydroxide.

The hypothesis of a reaction between salt molecules and pectin is supported by the general analogy between the reactions described here and those of the sugar group. The constitution of the so-called saccharates and the analogous glucose compounds with the alkaline earths is doubtful, but formulae of the type assumed here for the pectin compounds, in which CaOH or its analogues replace a hydrogen atom, e.g. $C_{12}H_{21}(CaOH)O_{11}$, $C_6H_{11}(CaOH)O_6$, are not improbable, and have been strongly supported by some investigators [see especially Lippmann, 1904, p. 1701]. Compounds with the alkalies are similarly formed, e.g. $C_{12}H_{21}KO_{11}$ [Tollens, 1914, p. 400]. The sugars are also known to form molecular compounds with sodium chloride and many other salts [Tollens, 1914, p. 404; Lippmann, 1904, p. 1323].

In the ordinary notation the equation

$$\frac{dx}{dt} = KC_1C_2(a-x)$$

represents the reaction of one molecule of pectin with e.g. the ions Ba and OH. Now the molecule of pectin is certainly large, and is made up, at least to a very great extent, of condensed carbohydrate groups; these groups will be largely independent of one another, and therefore it is probable that for the purposes of this equation they may be regarded as independent molecules. The equation will therefore represent the rate of reaction of a carbohydrate group with the ions Ba and OH, rather than that of the whole complex molecule.

The rate of reaction of pectin with barium hydroxide (Table VI) increases with the concentration of the pectin while this is very small; at higher concentrations the rate becomes stationary, and it finally decreases. There is probably more than one cause for this behaviour. The fact that the rate

of reaction decreases much more rapidly for strontium than for barium suggests that absorption plays an increasing part, and that for the reason given on page 558, gelatinisation and precipitation become less and less coincident as the concentration of pectin increases. But besides this other factors may be brought into play; unchanged pectin probably acts to impede gelatinisation, and there is good reason to believe that the degree of dispersion of pectin decreases with the concentration, so that the active mass will increase less rapidly than the concentration; the importance of this latter factor in dilute solution is however entirely problematical.

Turning now to the absorption compounds which gelatinised pectin forms with the alkaline earths, it must be remarked that the term "absorption compound" has been used in its most general sense to denote a compound of continuously varying composition. Reference to the curves in Fig. 4 shews that the absorption curves are straight lines cutting the y axis at varying points. The simplest explanation of a straight line absorption curve is that of solid solution. Surface adsorption is precluded both by the character of the curve and by the slowness with which the condition of equilibrium is reached at small concentrations, which again favours the hypothesis of a solid solution. If surface adsorption occurs at all it must be to a very small extent.

If V be the volume of the jelly; A the amount of alkaline earth absorbed; N the partition coefficient of alkaline earth between jelly and solution, we have for a solid solution at concentration C

$$\frac{CV + A}{CV} = N, \quad \text{i.e. } A = CV(N-1),$$

which gives a straight line when V is constant.

It has been shewn that a true equilibrium is not reached under the conditions of the experiments, and that absorption continues for days, probably owing to a slow process of diffusion. This has been taken to mean that the apparent equilibrium is due to saturation at the surface; V will consequently be a function of the surface area. Now $V(n-1) = \tan\theta$ if θ is the angle the absorption curve makes with the axis of x, and it will be seen by comparing curves I and II Fig. 4 that $2\tan\theta_2$ is greater than $\tan\theta_1$; accordingly $\frac{V_2}{2}$ is greater than V_1, i.e. the specific surface increases as the concentration of the pectin decreases.

The absence of surface adsorption is important, since in this case the point at which the straight line cuts the y axis will give the composition of

the compound of alkaline earth and pectin. This is confirmed by the convergence of the curves for calcium, strontium and barium (Fig. 4 III, IV, V) which approximately meet at a point on the y axis. These curves give the absorption for 10 cc. of pectin solution (c) in 100 cc.; the solutions were therefore very dilute, and the convergence to a point is to be attributed to this fact. At higher concentrations it is most unlikely that this occurs, and some experimental evidence was obtained on this point, but it needs further investigation.

Taking 1·9 (2·2 – 0·3 for the acidity) as combining with 10 cc. of pectin solution containing approximately 2·5 grms. of dry pectin per litre, we have 330 as the weight of pectin combining with the equivalent of alkaline earth. This figure is certainly too high since the specimen of pectin was not pure, and no allowance was made for the weight of ash. It may be compared with the formula $6\,(C_{10}H_{18}O_9) + O$ suggested by Tollens. The weight of the $C_{10}H_{18}O_9$ group is 282.

It is well known that the alkaline earths are easily soluble in sugar solution, and cane sugar has been shewn to form a series of solid solutions with lime [Cameron and Patten 1911], so that in this case again the resemblance of pectin to the members of the sugar group is preserved. It has been shewn that calcium chloride as such is not absorbed by pectin; it merely serves to increase the absorption of the calcium hydroxide, and a similar effect is obtained with barium chloride and barium hydroxide. This may be a salting out process, due to decreased dissociation; but it seems more probable, at any rate in the case of calcium, that the chloride increases the solubility of the hydroxide in pectin as it does in water [Cameron and Bell 1907], but to a greater extent, thus increasing the value of the partition coefficient n.

Theory of gelatinisation of pectin.

References to the theory of gelatinisation have been hitherto avoided, and it has been assumed that alterations of physical or chemical properties may suffice to bring this process about, without reference to the mechanism of the change. This now remains to be considered, and it will be necessary, as a preliminary, to discuss shortly the current theories of gelatinisation [see the historical introduction, Bachmann 1912].

Until very recently jellies have been almost universally supposed to consist of two phases separated by a process of desolution during the solidification of the original sol. According to this theory where desolution results in

gelatinisation the one phase separates in droplets, which remain suspended in the continuous phase, and afterwards cohere, and in most cases solidify, forming the framework of the gel. Hardy [1900] observed this process for mixtures of alcohol, water and gelatin, and he found it possible, by suitably varying the conditions of experiment, to obtain droplets of almost ultra-microscopic size.

Hardy's work gave fresh confirmation to the widely accepted honeycomb theory of gel structure due to Bütschli's classical researches on the structure of gelatin [1898], a theory which was also supported by Quincke's theoretical investigations, and by the authority of van Bemmelen, who used it for the interpretation of his later work ; it was however adversely criticised in some quarters ; notably by Pauli [1902] who regarded the structure observed by Bütschli in coagulated gelatin as a secondary structure due to the coagulation, and not as preëxisting in the uncoagulated jelly. To establish this point he investigated the action of the coagulating agent, and arrived at the conclusion that essential differences exist between the processes of gelatinisation and coagulation, a conclusion very important for the theory of these processes. He shewed that the one process could be accelerated by means which caused retardation in the other ; that e.g. grape sugar added to gelatin accelerates gelatinisation and raises the temperature of solidification, while it hinders precipitation ; and that the independence of the two processes was also proved by the fact that coagulation could take place as well in solid as in liquid gelatin. He also pointed out certain discrepancies between the results of Bütschli's theory and experiment, and he concludes that the " species of coagulation " worked by chromic acid, alcohol, etc. in solidified gelatin must be regarded as a true coagulation, essentially different from the preceding process of solidification, that the honeycomb structure observed in the coagulation is secondary, and that those appearances in uncoagulated gelatin which Bütschli regarded as due to this structure must be looked upon rather as the result of imperfect homogeneity.

Pauli's conclusions did not meet with general acceptance, and the theory of Bütschli held its ground until the introduction of ultra-microscopic methods of research allowed the application of a conclusive test. By this means Bachmann [1912] has shewn that gelatin jellies possess a far more complicated structure than the theory of Bütschli would allow, being built up of microns, sub-microns, and probably still finer elements. Bachmann points out also that the jellies prepared by Hardy are of a very different character from those of gelatin, being soft, white and opaque, rather than clear and stiff. Such

difference in character may well denote an altogether different method of formation.

The continuity of properties which has so often been observed between sol and gel has led some investigators to regard gels as sols of greatly increased vicosity, but vicosity alone cannot account for their properties. Hatschek [1911] has pointed out that gels differ from highly viscous substances, such as pitch, in their inability to flow, as do these, however slowly. Any theory of gels must take account of this rigidity, as also of the heterogeneity of structure which they exhibit, which is usually of the same order as that of the sol from which they are derived.

Bütschli's theory of the structure of gels replaced an older theory due to Nägeli; this "micellar theory" contemplated the gel as consisting of molecular aggregations or *micelle* of varying degrees of complexity, separated by films of water and attracted together more or less strongly according to the thickness of the intervening film. The breakdown of Bütschli's theory has brought about a return to more or less similar conceptions [cf. Zsigmondy 1912, p. 72]. These afford a fairly satisfactory explanation of the coagulating effect of change of potential on inorganic sols, but are less successful where organic sols are concerned, the gelatinisation of which takes place at much smaller concentrations, and appears to be largely independent of electrical conditions, and there is some tendency at present to regard gelatinisation in these as due to the properties which organic sols share with true solutions, rather than to those which they possess in common with the suspensoids, i.e. as a process akin to crystallisation, rather than as a process of aggregation due to a change in the conditions of suspended particles. It must be recognised however that our present ignorance of the nature of aggregation processes makes this a somewhat provisional distinction. Procter [1911] regards the sol of gelatin in this light, and considers its gelatinisation to be due to the formation of a molecular network, while the work of Pauli [1913] on protein solutions has shewn the relationship of the aggregation processes which take place in these to the phenomena of crystallisation.

It has been shewn that the rate of gelatinisation of pectin solutions may be regarded as determined by rate of chemical combination; this at once suggests a crystallisation process. It is of course to be expected that chemical change in suspended particles might cause aggregation and precipitation, but it is difficult to conceive that the rate of aggregation would be directly proportional to the rate of chemical change. Those aggregation processes, the rates of which have been studied, have been shewn to depend upon the

adsorption of oppositely charged ions [cf. Freundlich and Ishizaka, 1913: Paine, 1912], and in these cases the rate of precipitation shews no simple relation to that of adsorption; also where aggregates separated by films of water are concerned, rate of diffusion must determine rate of chemical action, and moreover, in the present case, the extreme dilution at which pectin gelatinises in alkaline solution makes any but a molecular hypothesis difficult of acceptance. Therefore here also the "crystallisation" hypothesis affords the simplest explanation of the facts.

During the last few years von Weimarn's [1911] work has thrown much light on the theory of gelatinisation, and since his theory is the most complete expression of the "crystallisation" theory, an attempt will be made to outline this in order to compare with it the results obtained for pectin jellies.

Von Weimarn regards inorganic jellies as concentrated highly disperse suspensoid solutions. He has himself prepared jellies from barium sulphate and other inorganic substances, and he has shewn that in general any substance under suitable conditions may be prepared in this form, the requisite conditions being to combine a sufficiently high degree of dispersion with a sufficiently high concentration. His theory of crystallisation, which is on the lines of the preceding theories of Lehmann and Tammann but permits of wider application, shews the relation of these jellies to substances in ordinary crystalline form. He regards crystallisation as the result of two independent processes (1) the formation of molecular aggregates by sudden concentration, and (2) the growth of centres by diffusion, either of molecules or of small aggregates. The first process gives rise to a suspensoid solution, and from this crystals are formed by the second process of diffusion. The rate of the first process, which von Weimarn takes as proportional to the relative supersaturation (i.e. the ratio of the quantity of substance capable of separating out to the solubility), determines the degree of dispersion; where this is great the degree of dispersion will be high. Now if the second process is impeded or inhibited, stable suspensoid solutions will arise, and this will evidently take place when the degree of dispersion is so high that the greater part of the substance present separates during the first process in the form of small molecular aggregates, for these will neither diffuse rapidly nor combine readily with one another, since their vector properties will be small. A stable suspensoid solution is therefore formed, when a substance separates from a solution whose relative supersaturation is very high; the greater the solubility the greater the relative supersaturation, and the higher the degree

of dispersion necessary for stability; concentrated suspensoid solutions will take the form of a jelly.

As a test of his theory von Weimarn prepared a number of disperse solutions by the admixture of solutions containing substances which mutually precipitate one another; e.g. barium sulphate was obtained at different degrees of dispersion by mixing solutions of barium chloride and magnesium sulphate at various concentrations. At the highest concentrations jellies of cellular structure were produced, since the reaction took place at the surface where the two solutions were in contact, and by maintaining the same degree of relative supersaturation with a lesser solubility (by alteration of the solvent) a jelly of network structure could be obtained.

In the application of this theory to organic sols, von Weimarn points out that between true solutions and disperse solutions an absolute continuity of properties exists, and that "the properties peculiar to the solutions of simple substances decrease in intensity as the molecular complexity increases." These complex solutions gelatinise readily because their molecules diffuse with difficulty, and with increase of concentration form groups practically incapable of overcoming the internal friction of the solvent, while cohesion is weak since the energy of the molecule is exhausted in its complicated internal structure. They form in fact disperse solutions, in which at small concentrations diffusion and vectorial cohesion are as much suppressed as in the highly concentrated disperse solutions of inorganic substances, and with a similar result. A lattice work is thus formed, the particles of which are bound together by vectorial forces, and which may therefore be entitled crystalline, but which will be soft and weak, and will be penetrated by the solvent, and greatly deformed [von Weimarn 1912].

The size and nature of the particles of which pectin solutions are formed have not been determined, but there is some reason to believe that the degree of dispersion decreases with increase of concentration, since the difficulty of filtration increases; only very dilute solutions can be filtered through hardened filter paper without separation of the dissolved pectin. The characteristics are those of an emulsoid, and the solutions may very probably consist of a suspension of aqueous pectin in a dilute pectin solution. In this case the reaction with the alkalies and the alkaline earths will take place in the solution and at the surface of the particles. As the reaction proceeds a supersaturated solution will be formed; this, as has been shewn, will solidify to a jelly at any concentration at which a molecular network can be produced. At very small concentrations where suspended pectin must be almost entirely

absent, the solution will remain supersaturated until practically the whole of the pectin present has been transformed, for the presence of a foreign substance will greatly impede both diffusion and cohesion, and will thus hinder the formation of a continuous network. As concentration increases the relative supersaturation previous to gelatinisation will increase, while the fraction of pectin transformed will decrease ; the amount separating out will thus increase with the concentration, but in a smaller ratio. These considerations afford an explanation of the positions of the points at which the curves in Fig. 4 cut the y axis, as also of the rate measurements in Table VI.

Summary.

(1) The rate of gelatinisation of pectin is not determined by the rate of diffusion nor by the rate of surface adsorption of the alkali or alkaline earths producing gelatinisation.

(2) It can be expressed as a velocity equation determined by the concentration of the reacting substances, when the initial concentration of pectin is constant.

(3) It is therefore regarded as determined by the rate of a chemical reaction—the substitution of H by BaOH, K etc.

(4) That a reaction of this kind takes place is supported by the fact that the constitution deduced for compounds of the alkalies and alkaline earths is strikingly analogous to that of the corresponding compounds of the mono- and di-saccharides. Similar molecular compounds with salts are probably also formed.

(5) Gelatinised pectin forms solid solutions with the alkaline earths which may be compared with the solid solutions of lime and cane sugar.

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